2B or Not 2B:
Capsular Wnt Activates Wnt-High Signaling to Maintain Adrenal Glomerulosa Identity

by

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DEDICATION

To all the patients, who are the reason for this work.

To my family, for all your unconditional support.

To my mother, who is a continual warrior.

To my loving wife, who always strengthens and encourages me.

To my son Asher, for the light you are and all you will be.

To the Triune God alone be all glory.

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LIST OF ABBREVIATIONS

ACA	adrenocortical adenoma
ACC	adrenocortical carcinoma
ACT	adrenocortical tumor(s)
ACTH	adrenocorticotropic hormone
AGP	adrenogonadal primordium
AIMAH	adrenocorticotropin-independent macronodular adrenocortical hyperplasia
AJ	adherens junction
AngII	angiotensin II
AS	aldosterone synthase
BMP	bone morphogenic protein
CCDC80	coiled-coil domain-containing protein 80
CD	Crohn’s disease
cKO	conditional knockout
CM	conditioned media
Co-IP	co-immunoprecipitation
CRC	colorectal cancer
CRH	corticotropin-releasing hormone
DRO1	downregulated by oncogenes 1

ECM	extracellular matrix
ENSAT	European Network for the Study of Adrenal Tumors
FAP	familial adenomatous polyposis
FDH	focal dermal hypoplasia
FEVR	familial exudative vitreoretinopathy
FGF	fibroblast growth factor
GEPIA	Gene Expression Profiling Interactive Analysis
GI	gastrointestinal
gKO	global knockout
GOF	gain-of-function
GSEA	Gene Set Enrichment Analysis
H&E	hematoxylin and eosin
HPA (axis)	hypothalamus-pituitary-adrenal
HSPG	heparin sulfate proteoglycan
IBD	inflammatory bowel disease
IF	immunofluorescence
IHC	immunohistochemistry
ISH	*in situ* hybridization
KO	knockout
LC-MS/MS	liquid chromatography/tandem mass spectrometry
LOF	loss-of-function
PA	primary aldosteronism
PCP	planar cell polarity (Wnt/PCP)

PPNAD	primary pigmented nodular adrenocortical disease
RAAS	renin-angiotensin-aldosterone system
scRNAseq	single-cell RNA sequencing
SERKAL (syndrome)	sex reversion, kidney, adrenal, lung dysgenesis
Shh	Sonic hedgehog
smISH	single-molecule in situ hybridization
Ssg1	steroid-sensitive gene 1
TCGA	The Cancer Genome Atlas
UC	ulcerative colitis
zF	zona fasciculata
zG	zona glomerulosa
zR	zona reticularis
ΔCat	*Akr1b7-Cre*; *β-catenin*$^{flox(ex3)}$

ABSTRACT

The adrenal glands are endocrine organs that produce steroid hormones essential for life. Steroidogenesis occurs in the adrenal cortex, a highly regenerative tissue that is organized in concentric zones of cells surrounded by a mesenchymal capsule. Diseases of the adrenal cortex include hypo-/hyperplasia, dysfunctional steroid hormone production, and carcinoma. Adrenocortical carcinoma (ACC) is a rare but highly metastatic malignancy that poses a high risk of recurrence and for which successful targeted therapy options are lacking. Therefore, there is great need for understanding the basic underlying cellular and molecular mechanisms that are involved in ACC and other adrenal diseases.

Long-term retained adrenocortical progenitor cells reside in the subcapsular zona glomerulosa (zG). The adrenal zG is a critical compartment of these undifferentiated progenitor cells as well as differentiated cells that produce aldosterone, an essential steroid hormone involved in physiological salt and water balance, that centripetally differentiate to replenish glucocorticoid-producing cells of the inner zona fasciculata (zF). Both progenitor and aldosterone-producing cell populations respond to Wnt/Beta-catenin signaling, a conserved paracrine pathway necessary for proper adrenocortical development and homeostasis. Wnt/Beta-catenin signaling is altered in 40% of ACCs and plays a role in several other adrenocortical diseases. Through our work, we have identified a capsular Wnt ligand that functions to activate and maintain Wnt-high activity

in the adrenal cortex that is necessary for zG cell identity and proper functional capacity. The focus of this dissertation is to define the role this capsular Wnt ligand, WNT2B, in adrenocortical Wnt/Beta-catenin signaling and to determine the consequences of its loss on adrenocortical homeostasis.

Here, we show that human patients with *WNT2B* loss, who exhibit chronic congenital diarrhea and subsequent hypovolemia, also have hypoaldosteronism that is compensated by excessive activation of the renin-angiotensin-aldosterone system (RAAS). To understand the mechanism by which WNT2B regulates zG cell aldosterone production, we developed a global *Wnt2b* knockout mouse model that exhibits near complete zG loss, indicating the necessity of WNT2B for proper zG development. Moreover, mice with conditional *Wnt2b* loss in adulthood have decreased adrenocortical cell proliferation and expression of progenitor cell markers, supporting a role for Wnt/Beta-catenin signaling in progenitor cell maintenance. *Wnt2b*-deficient mice also exhibit dampened Wnt-high activity in the adrenal zG. Finally, mice lacking *Wnt2b* exhibit downregulation of *Cyp11b2*, the terminal enzyme in aldosterone synthesis, but have comparable plasma aldosterone levels to controls. We then show that *Wnt2b* loss results in elevated plasma renin, which acts to compensate for the reduced steroidogenic capacity of differentiated zG cells of the adrenal cortex.

Taken together, our studies illuminate the role of a newly discovered adrenal WNT ligand essential for the growth, maintenance, and physiological function of the adrenal zG. Our work highlights the underlying mechanisms of an adrenal defect in patients with LOF *WNT2B* mutations which we hope will inform our understanding and treatment of their disease as well as the molecular underpinnings of Wnt-active ACC.

CHAPTER 1 Wnt/β-catenin Signaling in Adrenocortical Biology

1.1 Disclosure of relevant publications

Portions of this work have been published:

Little III DW, Dumontet T, LaPensee CR, Hammer GD. β-catenin in adrenal zonation and disease. *Molecular and Cellular Endocrinology.* 2021 Feb 15. 522:111120-111159.

Portions of this work are being prepared for publication:

Little III DW*, Borges KS*, Basham KJ, Azova S, O'Connell AE, Dumontet T, LaPensee CR, Breault DT, Hammer GD. WNT2B is essential for adrenal glomerulosa identity and function. In preparation. *co-first author

1.2 Introduction

The Wnt signaling pathway is a critical mediator of the development and maintenance of several tissues. The adrenal cortex is highly dependent upon Wnt/β-catenin signaling for proper zonation and endocrine function. Adrenocortical cells emerge in the peripheral capsule and subcapsular cortex of the gland as progenitor cells that centripetally differentiate into steroid hormone-producing cells of three functionally distinct concentric zones that respond robustly to various endocrine stimuli. Wnt/β-catenin signaling mediates adrenocortical progenitor cell fate and tissue renewal to maintain the gland throughout life. Aberrant Wnt/β-catenin signaling contributes to various adrenal

disorders of steroid production and growth that range from hypofunction and hypoplasia to hyperfunction, hyperplasia, benign adrenocortical adenomas, and malignant adrenocortical carcinomas. Great strides have been made in defining the molecular underpinnings of adrenocortical homeostasis and disease, including the interplay between the capsule and cortex, critical components involved in maintaining the adrenocortical Wnt/β-catenin signaling gradient, and new targets in adrenal cancer. This review seeks to examine these and other recent advancements in understanding adrenocortical Wnt/β-catenin signaling and how this knowledge can inform therapeutic options for adrenal disease.

1.3 A brief history of Wnt signaling

Wnt signaling was first discovered in *Drosophila* in the 1970s with the field rapidly expanding with several landmark studies in mice and humans. Loss of the *wingless* gene, as its name suggests, was responsible for a wingless phenotype due to altered polarity and wing morphogenesis in *Drosophila* (Sharma and Chopra, 1976). Within a decade, its mouse homolog, called *integration site 1* (*Int1*), was discovered in a murine virus that resulted in sporadic mammary gland tumorigenesis (Nusse and Varmus, 1982). Mouse *Int1* was found to be nearly identical to its human homolog, a mammary gland proto-oncogene deemed *WNT1* (*wingless/Int1*) (van Ooyen and Nusse, 1984; Nusse et al., 1990). *WNT1* and its associated pathway became the focal point of a vast array of

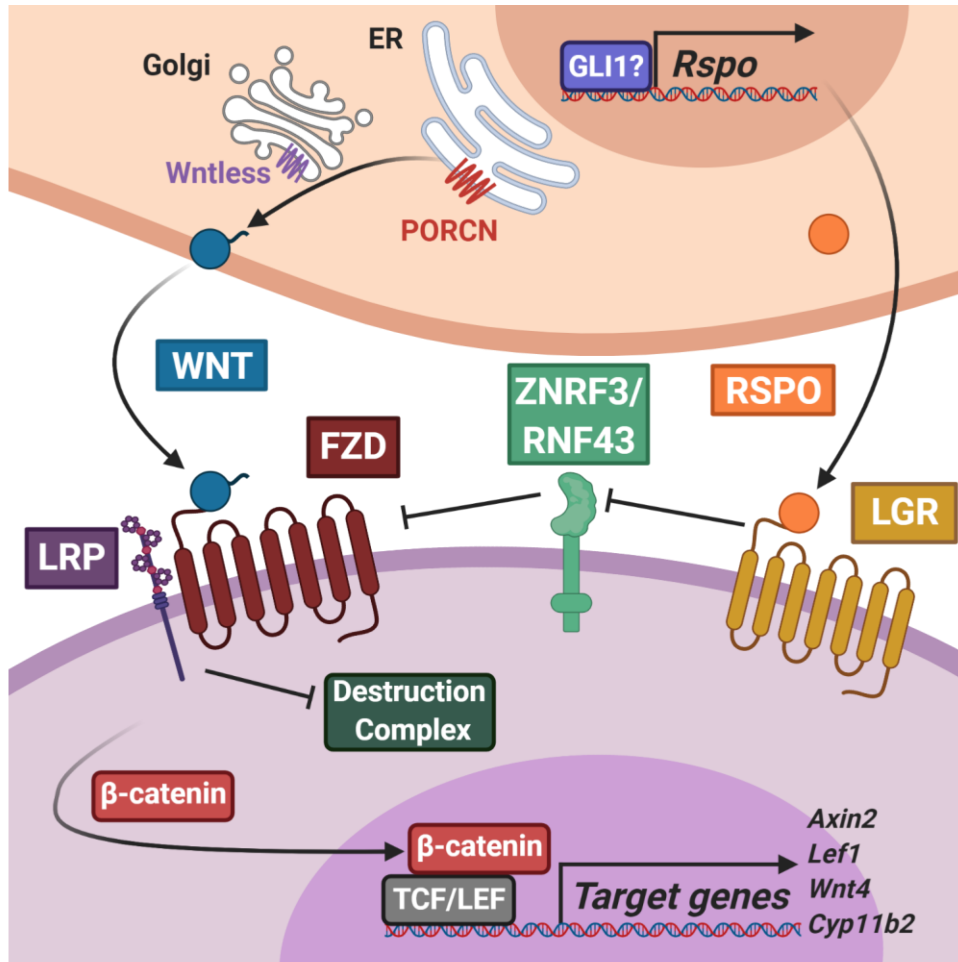


Figure 1.1. Canonical Wnt signaling activates β-catenin-dependent transcription
(Top) WNT ligands are palmitoylated by Porcupine (PORCN) in the endoplasmic reticulum (ER) and joined by Wntless in the Golgi apparatus for secretion from WNT-producing cells. R-spondin (RSPO) ligands are also produced and secreted alongside WNT ligands. (Bottom) WNT ligands bind to Frizzled (FZD) and LRP receptors, which are marked for degradation and turnover by ZNRF3. RSPO ligands bind to LGR to inhibit ZNRF3 and potentiate WNT-activated signaling. Activation of FZD/LRP receptors recruits components of the β-catenin destruction complex to the cell membrane. In turn, β-catenin is stabilized and translocates to the nucleus where it co-activates target gene expression with TCF/LEF transcription factors. Created with BioRender.com.

biological research, spanning from *Drosophila* development to human cancer, over the next five decades.

WNT1 was first to be discovered among the WNT ligands, a family of 19 paralogs that code for secreted glycoproteins that bind to Frizzled (FZD) receptors and activate downstream intracellular signaling (Nusse et al., 1991). WNT ligands activate two primary intracellular signaling cascades defined by β-catenin-independent and β-catenin-dependent transcriptional activation (reviewed in Wiese et al., 2018 and Zhan et al.,

2017). Wnt signaling independent of β-catenin, termed non-canonical, comprises both the planar cell polarity (Wnt/PCP) and calcium (Wnt/Ca^{2+}) pathways. The canonical Wnt pathway, hereafter referred to as Wnt/β-catenin, involves activated β-catenin-induced target gene expression **(Figure 1.1)**. Wnt/β-catenin signaling is co-regulated by a group of proteins known as the destruction complex, containing GSK3β, APC, and AXIN2, among others. In the absence of WNT ligands, cytoplasmic β-catenin is targeted for degradation through phosphorylation by GSK3β of the destruction complex. Upon FZD activation by WNT ligands, components of the destruction complex are recruited to the cell membrane, stabilizing β-catenin and allowing it to translocate to the nucleus where it serves as a coactivator of TCF/LEF-mediated transcriptional activation. An additional level of Wnt/β-catenin signaling modulation is provided by ZNRF3, an E3 ubiquitin ligase that targets FZD receptors for internalization and degradation. Moreover, R-spondin (RSPO) ligands bind to LGR receptors, leading to ZNRF3 turnover and enhancing Wnt/β-catenin signaling activation through FZD receptor bioavailability.

Numerous studies have implicated Wnt/β-catenin signaling as a critical regulator of organ development and homeostasis through specific regulation of tissue-specific stem/progenitor cell populations, including those in the adrenal cortex. Such a role in the adrenal was first supported by observations in patients with familial adenomatous polyposis (FAP) who harbored germline mutations in the *APC* gene (Smith et al., 2000; Marchesa et al., 1997). In addition to early onset colonic polyps, patients with *APC* mutations had an increased incidence of benign and malignant adrenocortical tumors (ACTs), suggesting that, in addition to contributing to carcinogenesis, β-catenin may be crucial for proper adrenal homeostasis (Gaujoux et al., 2010).

The adrenal glands are bilateral organs which lie atop the kidneys and produce steroid hormones essential for various physiological functions, including salt balance, glucose metabolism, and sexual maturity. The adrenal is composed of an outer mesenchymal capsule and underlying cortex comprised of histologically distinct concentric zones responsible for the synthesis of three classes of steroid hormones **(Figure 1.2)**. The peripheral subcapsular zona glomerulosa (zG) is marked by small, compact cell clusters responsible for producing aldosterone, a mineralocorticoid essential for maintaining salt and water balance (Arnold, 1866). Centripetal to the zG lies the zona fasciculata (zF), comprised of fascicules of larger cells that produce cortisol in primates (corticosterone in rodents). Primate but not rodent adrenals also have a zona reticularis (zR) between the zF and central medulla that produces sex steroids. The centrally-located medulla is a functionally and histologically distinct coalescence of catecholamine-producing cells derived from the neural crest (Le Douarin and Teillet, 1974; Furlan et al., 2017).

Multiple groundbreaking studies have provided key insights into the importance of Wnt/β-catenin signaling not only in tumorigenesis but also in the development and maintenance of the adult adrenal cortex where Wnt-responsive progenitor cells of the zG play a critical role in renewal of the cortex throughout life (Heikkilä et al., 2002; Kim, A.C. et al., 2008; Berthon et al., 2010; Ingle and Higgins, 1938; King et al., 2009; Freedman et al., 2013). In this chapter, I will highlight exciting new studies that have added tremendous depth to our understanding of Wnt/β-catenin signaling in adrenocortical zonation, progenitor cell biology, and adrenal cancer. The roles of Wnt pathway components and antagonists, including RSPO3, ZNRF3, PKA, and β-catenin itself, in adrenocortical

zonation, architecture, and steroidogenesis will be discussed. The role of Wnt/β-catenin signaling in adrenocortical development, homeostasis, and regeneration will also be reviewed. Emphases on the molecular interplay between the capsule and cortex, the newly discovered adrenocortical Wnt/β-catenin signaling gradient, and continued questions regarding zG cell-specific transcriptional programs and potential cell(s) of origin in adrenal disease will be made throughout. Finally, I will outline the great advancements made in understanding the contribution of Wnt/β-catenin signaling to adrenal tumorigenesis, especially adrenocortical carcinoma (ACC), with implications for new targeted therapeutic options for patients.

1.4 Adrenal cortex: zonation and renewal

The adrenal cortex contains concentric zones of steroidogenic cell populations that are histologically and functionally distinct **(Figure 1.2)**. Steroidogenic cells of the adrenal cortex are marked by expression of steroidogenic factor 1 (*Sf1*/*Nr5a1*/*Ad4bp*), an orphan nuclear receptor that acts as a master regulator of steroidogenic enzyme gene expression and cell fate (Morohashi et al., 1992; Lala et al., 1992). SF1 is essential for gland development and function throughout life as evidenced by adrenal hypoplasia and hypofunction in the setting of mice and human patients with loss-of-function (LOF) mutations in *SF1* (Morohashi et al., 1992; Ingraham et al., 1994; Sadovsky et al., 1995; Luo et al., 1994; Hammer et al., 1999; Val et al., 2003; Beuschlein et al., 2002; Achermann et al., 1999). Immediately below the overlying capsule lies the zG, consisting of dense cell clusters historically referred to as glomeruli due to a similar appearance to the glomeruli of the kidney nephron (Zelander, 1957; Cater and Lever, 1954). Each

glomerulus is a biological rosette, a round grouping of differentiated steroid-producing cells and undifferentiated non-steroid-producing progenitor cells that is surrounded by laminin β1 (Leng et al., 2020). Steroid-producing cells within zG rosettes are marked by aldosterone synthase (AS, or CYP11B2), the terminal enzyme in aldosterone production that is regulated by plasma angiotensin II (AngII), sodium (Na^{2+}), potassium (K^{+}), and calcium (Ca^{2+}) in primates and rodents (Ogishima et al., 1992; Imai et al., 1992; Mitani et al., 1994; Kakiki et al., 1997; Ye et al., 2003; Clyne et al., 1997; Fakunding et al., 1979; Guagliardo et al., 2020). The zG makes up only a small portion of the cortex, which largely consists of zF. The zF is organized in long fascicules of larger *Cyp11b1*-expressing cells responsible for producing corticosteroids in response to ACTH as part of the hypothalamus-pituitary-adrenal (HPA) axis.

Foundational experiments in rats showed that enucleated adrenals with a remaining zG and capsule renew over time, providing evidence that the capsule and zG together are a crucial progenitor cell compartment responsible for long-term cortical cell replenishment (Greep and Deane, 1949). Lineage tracing studies extended these findings by showing that the underlying zF is replenished by zG cells, which differentiate from aldosterone-producing to corticosteroid-producing cells that are centripetally displaced inward toward the medulla to eventually die at the corticomedullary boundary (Wright et al., 1973; Zajicek et al., 1986; King et al., 2009; Freedman et al., 2013). When

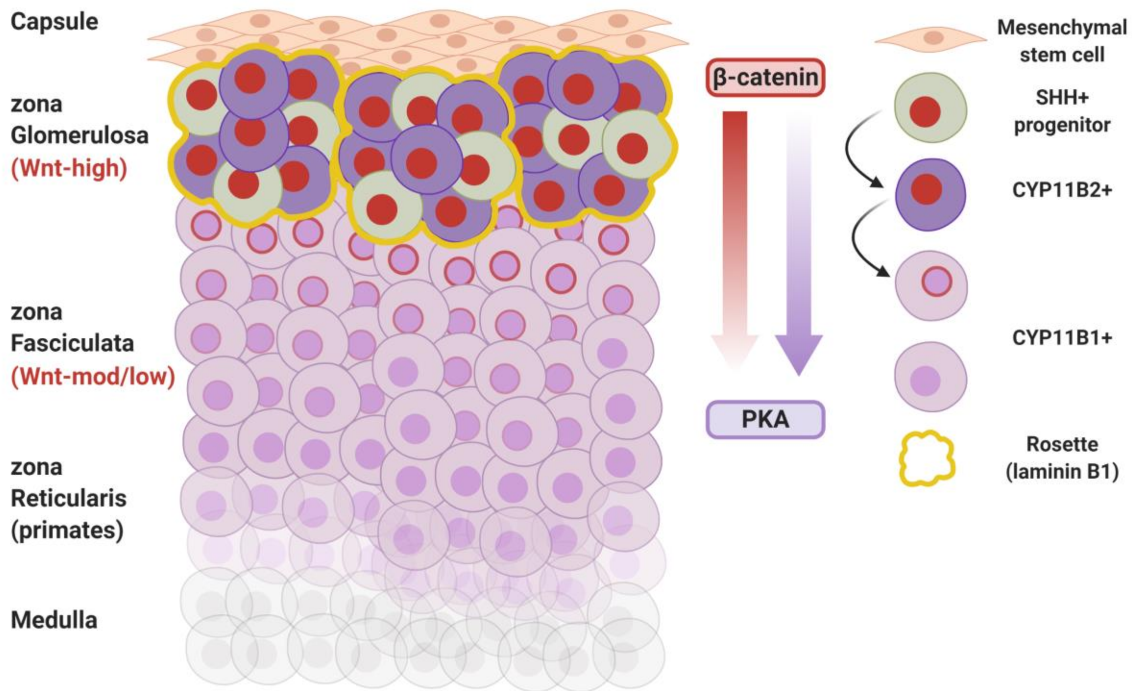


Figure 1.2. The adrenal cortex is maintained through centripetal differentiation
(Left) The adrenal gland contains an outer mesenchymal capsule, middle steroidogenic cortex, and inner medulla. The cortex is composed of concentric zones that are histologically and functionally distinct. Below the capsule lies the zona glomerulosa (zG), a progenitor cell compartment organized into rosettes (gold outline) of undifferentiated SHH-expressing/CYP11B2-negative (green) and aldosterone-producing SHH-negative/CYP11B2-expressing (dark purple) cells. Cells of the zG differentiate and centripetally replenish corticosteroid-producing CYP11B1-expressing cells of the zona fasciculata (zF; light purple). Adrenocortical cells eventually die at the corticomedullary boundary. (Middle) Wnt/β-catenin signaling exists in a centripetal gradient in the adrenal cortex. The adrenal zG is marked by Wnt-high activity, indicated by red nuclei. As zG cells differentiate, β-catenin is inhibited by PKA signaling (purple nuclei), allowing for zG-to-zF transdifferentiation and corticosteroid production. Created with BioRender.com.

aldosterone-producing zG cells were permanently labeled with GFP utilizing a Cre recombinase lineage tracing system under the control of the *Cyp11b2* promoter (*AS-Cre*; *mTmG*), zG descendants were observed within the cortex 5 weeks after birth and completely replenished the cortex by 12 weeks (Freedman et al., 2013). In addition to a steroid-producing lineage in the zG, a non-steroid-producing SF1-expressing progenitor cell population marked by SHH also resides in the zG. These self-renewing SHH-expressing progenitor cells serve to replenish steroid-producing cells of the cortex by differentiating into CYP11B2-expressing or CYP11B1-expressing cells of the zG or zF, respectively (King et al., 2009; Huang et al., 2010; Walczak et al., 2014). Interestingly, SHH ligands secreted by zG progenitor cells signal to the overlying capsule, where they bind to Smoothened (SMO) receptors, inhibiting Patched (PTCH) and activating intracellular GLI1-mediated gene transcription (Nuesslein-Volhard and Wieschaus, 1980; King et al., 2009). Finally, the adrenal capsular *Gli1*-expressing cell lineage also contributes (albeit at a lower frequency) to cortical cell replenishment over time, particularly during establishment of the adult cortex following encapsulation of the fetal gland and under various injury paradigms (King et al., 2009; Finco et al., 2018; Grabek et al., 2019). These data collectively define the capsular-cortical unit that makes up the adrenocortical progenitor cell niche.

The adrenocortical progenitor cell niche involving the capsule and zG is regulated by both paracrine and endocrine signals that govern the balance between progenitor cell self-renewal and differentiation into steroid-producing cells to consistently maintain the adrenal cortex over time. Wnt signaling is an essential paracrine pathway involved in zG maintenance and adrenocortical homeostasis. β-catenin, the main intracellular effector of

canonical Wnt signaling, is highly expressed in the zG in mice, although recent evidence has shown that β-catenin activity exists in a centripetal zG-to-zF gradient (Kim, A.C. et al., 2008; Basham et al., 2019). β-catenin regulates both the proliferation and steroidogenic activity of zG cells to maintain the progenitor cell population while also activating *Cyp11b2* expression and aldosterone production in cells that eventually replenish the zF (Kim, A.C. et al., 2008; Berthon et al., 2010; Freedman et al., 2013; Pignatti et al., 2020). Wnt/β-catenin signaling activates the expression of downstream target genes *Lef1* and *Axin2* in the zG, the latter of which also marks cells whose descendants repopulate the inner cortex over time (Filali et al., 2002; Jho et al., 2002; Finco et al., 2018; Grabek et al., 2019). Furthermore, fluorescence-activated cell sorting (FACS) experiments on GFP-labeled Wnt-responsive zG cells identified a population of SHH-expressing/CYP11B2-negative progenitor cells (King et al., 2009; Walczak et al., 2014). Notably, developmental SF1-Cre-mediated ablation of *Shh* in mice resulted in adrenocortical hypoplasia that continued into adulthood (Ching and Vilain, 2009). Together, these studies demonstrate that the zG is a heterogeneous progenitor cell compartment that is maintained by active Wnt/β-catenin signaling. However, the underlying cell-specific β-catenin-dependent mechanisms that regulate zG cell fate, as well as zonal identity and morphology, are not yet fully understood.

1.5 Wnt/β-catenin signaling in adrenal zonation

Following the discovery of adrenal tumors in FAP patients harboring *APC* mutations, numerous studies focused on the role of β-catenin in adrenal biology. Mice harboring adrenal-specific GOF β-catenin presented with adrenocortical hyperplasia and

tumorigenesis later in life, while reciprocal studies showed that loss of β-catenin in the adrenal resulted in adrenocortical hypoplasia and late-stage failure of the gland (Berthon et al., 2010; Heaton et al., 2012; Kim, A.C. et al., 2008). These seminal findings led to further implementation of mouse models to study the underlying role of Wnt/β-catenin signaling in normal adrenocortical development and homeostasis. The following sections will outline Wnt/β-catenin-regulated adrenocortical zonation and homeostasis in the adult gland. The oncogenic role of β-catenin and other Wnt pathway components will be discussed in more detail in subsequent sections.

1.5.1 Capsular signaling in adrenocortical β-catenin activation

Adrenocortical Wnt/β-catenin activity is highest in the subcapsular zG, which contains both steroid-producing CYP11B2-expressing/SHH-negative and non-steroid-producing CYP11B2-negative/SHH-expressing progenitor cells (Kim, A.C. et al., 2008; Freedman et al., 2013; Walczak et al., 2014). Importantly, constitutive β-catenin activity results in zG-restricted hyperplasia in mice, suggesting that β-catenin induces proliferation in a zone-specific manner (Berthon et al., 2010; Pignatti et al., 2020). In addition to active β-catenin, Wnt-responsive cells are detected by expression of *Lef1*, the main nuclear β-catenin binding partner in the adrenal cortex, and the classic Wnt/β-catenin target genes *Axin2* and *Apc*, whose protein products are essential for negative regulation of β-catenin through the destruction complex (Filali et al., 2002; Ikeda et al., 1998; Amit et al., 2002; Lee et al., 2003; Xing et al., 2004; reviewed in Kimelman & Xu, 2006). WNT ligands, a family of 19 secreted glycoproteins, activate β-catenin by recruiting members of the destruction complex to the cell membrane (Nusse et al., 1991; reviewed in MacDonald et al., 2009). Of the WNT ligand family, *Wnt4* was detected in the Wnt-

responsive adrenal zG by *in situ* hybridization (ISH), suggesting that it at least in part regulates Wnt/β-catenin signaling in the cortex (Heikkilä et al., 2002). However, loss of *Wnt4* in mice resulted only in decreased aldosterone production and thus a physiological but not a morphological defect at 12 weeks of age (Heikkilä et al., 2002; Vidal et al., 2016). These observations led to the prediction that other ligands expressed in the capsule and/or cortex may be regulating β-catenin activity in the adrenal zG (Walczak et al., 2014). Indeed, ISH studies have identified the expression of *Wnt5a* and *Wnt11* in the cortical periphery and *Wnt2b* in the capsule (Lin et al., 2001; Pietila et al., 2016; Basham et al., 2019; Lako et al., 1998; Drelon et al., 2015). **The role of capsule-derived WNT2B in the adrenal cortex comprises the bulk of my thesis work and is discussed in detail in Chapter 2.**

The involvement of capsule-derived WNT ligands in adrenocortical Wnt/β-catenin signaling is supported by studies on R-spondin (RSPO) proteins, a family of secreted ligands (RSPO1-4) that potentiate Wnt/β-catenin signaling in both human and mouse (Chen et al., 2002; Kamata et al., 2004; Kim, K.A. et al., 2008). In the absence of RSPO ligands, FZD receptors are marked for internalization and degradation by ZNRF3/RNF43 transmembrane ubiquitin ligases (Chen et al., 2013; reviewed in Lau et al., 2014). When present, RSPO ligands bind to one of three leucine-rich repeat-containing G-protein coupled receptors (LGR4/5/6), coupling them with and subsequently neutralizing ZNRF3/RNF43. Thus, RSPOs inhibit negative regulators of Wnt/β-catenin signaling, increasing WNT receptor availability and ligand binding on the cell membrane **(Figure 1.1)**. The potentiation of Wnt/β-catenin signaling by RSPO ligands and the resultant increase of FZD receptor availability on the cell membrane through ZNRF3/RNF43

inhibition has proven to be essential for proper development and maintenance of several tissues. Recently, *in vitro* and *in vivo* studies have revealed that RSPO2/3, but not RSPO1/4, can potentiate Wnt/β-catenin signaling independent of LGR receptors through binding of heparin sulfate proteoglycans (HSPGs), a mechanism previously thought to act outside the scope of Wnt signaling (Lebensohn and Rohatgi 2018). Wnt/β-catenin signaling in the developing limb was unperturbed in mice lacking all three LGR receptors (*Lgr4*/*5*/*6* triple KO), suggesting that RSPO2/3 but not RSPO1/4 ligands continue to mediate ZNRF3/RNF43 turnover even in the absence of their receptors (Szenker-Ravi et al., 2018). Although LGR4 is expressed in the mouse adrenal cortex and would be predicted to elicit downstream RSPO-dependent turnover of ZNRF3/RNF43, this receptor-dependent activity of in the adrenal cortex has not yet been confirmed (Yi et al., 2013; Mazerbourg et al., 2004).

The necessity of *Rspo3* in adrenocortical zonation and zG identity was anticipated by single molecule *in situ* hybridization (smISH) that revealed the expression of *Rspo3* exclusively in the adrenal capsule (Vidal et al., 2016). Inducible capsule-restricted GLI1-CreERT2 and ubiquitous CAG-CreERT drivers were used to delete *Rspo3* in adrenal capsule in mice, which exhibited decreased adrenocortical Wnt/β-catenin activity and zG loss. Interestingly, *Wnt4* was downregulated in the adrenal cortices of *Rspo3* KO mice, suggesting that a capsular WNT ligand is required for zG maintenance. Indeed, SF1-Cre-driven *Wnt4* loss recapitulated *Cyp11b2* downregulation that has been previously shown but not the zG loss observed in *Rspo3* KO mice (Heikkilä et al., 2002; Vidal et al., 2016). Together, these data suggest that adrenocortical β-catenin activity is primarily dependent upon capsular RSPO3 and WNT ligands to maintain both the structure and function of

the adrenal zG—a critical progenitor cell compartment—and overall zonation of the adrenal cortex. The hypothesis that capsule-derived WNT ligands act alongside RSPO3 is supported by the secretion of RSPO and WNT ligands from underlying mesenchymal cells of the intestine that activate Wnt/β-catenin signaling and proliferation in neighboring stem cells to maintain the intestinal stem cell crypt (Valenta et al., 2016). Indeed, work presented in this dissertation highlights the role of WNT2B in activating adrenocortical Wnt/β-catenin signaling **(Chapter 2)**.

1.5.2 Adrenocortical Wnt/β-catenin signaling gradient

Historically, adrenocortical Wnt/β-catenin activity was restricted to the zG as observed in reporter mice (Walczak et al., 2014). However, newer, more sensitive molecular techniques have revealed a centripetal Wnt/β-catenin signaling gradient in the zG and upper zF that is guided by ZNRF3 (Basham et al., 2019). Recent work through both the European Network for the Study of Adrenal Tumors (ENSAT) and The Cancer Genome Atlas (TCGA) identified *ZNRF3* as a commonly deleted gene in ACC (detailed in 6.2; Assié et al., 2014; Zheng et al., 2016). ZNRF3 is an E3 ubiquitin ligase that labels FZD receptors for degradation to negatively regulate Wnt/β-catenin signaling (Hao et al., 2012; Koo et al., 2012). Deletion of *Znrf3* in the mouse adrenal cortex resulted in remarkable adrenocortical hyperplasia of cells in the upper zF, demonstrating for the first time the importance of this Wnt/β-catenin antagonist in mediating proper adrenocortical homeostasis and organization (Basham et al., 2019). Together with observations in the context of *Rspo3* loss by Vidal et al., Basham et al., provided corroborating evidence for an RSPO3/LGR4/ZNRF3 signaling mechanism from the adrenal capsule to the cortex

that mediates Wnt/β-catenin signaling and cortical zonation (Vidal et al., 2016; Basham et al., 2019).

Intriguingly, although *Znrf3* was expressed throughout the entire mouse adrenal cortex, the expansion of cells of the upper zF in *Znrf3*-null mice suggested that ZNRF3 is not primarily active in the zG, which remained normal (Basham et al., 2019). ZNRF3 activity thus contrasted that of β-catenin, which is most highly active in the zG and moderately active in the upper zF. Commensurate with the active β-catenin gradient was a *Wnt4* expression gradient, suggesting that high WNT4 levels may reinforce Wnt/β-catenin activity in the zG. Furthermore, *Znrf3*-null mice exhibited remarkably larger cortices than their control littermates due to expansion of the Wnt-moderate cell population of the upper zF that failed to inhibit β-catenin activity, although corticosterone levels were normal (Basham et al., 2019). The dependence of the observed hyperplasia on Wnt/β-catenin activity was demonstrated by β-catenin heterozygosity or *Porcn* loss, each of which partially rescued the effects of *Znrf3* loss. However, it is important to note that *Porcn* loss was restricted to the cortex, allowing for any potential intact WNT ligand processing and secretion from the capsule to blunt the rescue in *Znrf3*/*Porcn* dKO mice. These results support an antagonistic relationship between cortical ZNRF3 and capsular RSPO3 in mediating adrenocortical Wnt/β-catenin signaling: capsule-derived RSPO3 inhibits ZNRF3 to potentiate Wnt/β-catenin activity in zG cells immediately juxtaposed to the capsule, whereas ZNRF3 centripetally inhibits Wnt/β-catenin-dependent zF cell proliferation (Basham et al., 2019; Vidal et al., 2016). Furthermore, these data suggest a transient, proliferative Wnt-moderate cell population under the zG-zF boundary that, in

light of the importance of cell proliferation in adrenal tumorigenesis and recurrent *ZNRF3* deletions in ACC, has probable implications in ACC to be described later in this chapter.

1.5.3 Negative regulation of β-catenin in transdifferentiation

Proper transdifferentiation of zG cells to zF cells is essential for functional maintenance of the adrenal cortex throughout life (Pignatti et al., 2020). Multipotent cortical cell types of the zG, including both non-steroid-producing progenitor (SHH-secreting) cells and aldosterone-producing (CYP11B2-expressing) cells, expectedly undergo various transcriptional and epigenetic changes during their transition to corticosteroid-producing zF cells. Indeed, Wnt/β-catenin activity is diminished as cortical cells are centripetally displaced deep into the zF, implicating the necessity of Wnt/β-catenin inhibition during zG-to-zF transdifferentiation (Basham et al., 2019). In addition to regulation by ZNRF3 in the upper zF, Wnt/β-catenin signaling is also potently inhibited by cyclic AMP/protein kinase A (cAMP/PKA) signaling in the zF (Drelon et al., 2016a; Novoselova et al., 2018). Adrenocortical cells express melanocortin receptor 2 (MC2R), the receptor for adrenocorticotropin hormone (ACTH) that is necessary for zF development and stimulation of corticosteroid production (Chida et al., 2007; Gorrigan et al., 2011; Cooray et al., 2008). Differentiated zF cells also express melanocortin receptor accessory protein (MRAP), an MC2R binding partner that is essential for intracellular cAMP/PKA signaling induction and is frequently mutated in familial glucocorticoid deficiency (FGD) (Cooray et al., 2008; Gorrigan et al., 2011; Metherell et al., 2005). Corticotropin releasing hormone (CRH) from the hypothalamus induces ACTH secretion from the pituitary gland. ACTH binds to the MC2R/MRAP complex on the surface of zF cells and activates an intracellular Gs-protein response. Consequently, increased

intracellular cAMP binds to the regulatory domain of PKA, freeing its catalytic domain and allowing it to translocate to the nucleus. PKA phosphorylates cAMP response element binding protein (CREB), a transcription factor that, together with MAPK-phosphorylated SF1, regulates the ACTH-dependent expression of multiple steroidogenic enzymes (Hammer et al., 1999; Winnay et al., 2006). Thus, upon ACTH binding in the adrenal cortex, zF cells are induced to proliferate and increase corticosteroid production. In turn, plasma corticosteroids inhibit further CRH and ACTH secretion from the hypothalamus and pituitary, respectively, in a typical endocrine negative feedback loop.

Loss of the PKA regulatory domain *Prkar1a*, leading to constitutive PKA signaling, results in downregulation of the Wnt/β-catenin pathway in ACTH-sensitive zF cells (Drelon et al., 2016b; Dumontet et al., 2018). In the nuclei of ACTH-bound cells, CREB inhibits expression of *Wnt4*. Consequently, levels of active β-catenin are decreased and Wnt/β-catenin target genes are downregulated, promoting a zF phenotype. This was further supported by ACTH treatment of *Sf1-Cre*; *Wnt4*$^{fl/fl}$ mice that resulted in significantly reduced expression of *Axin2* compared to untreated animals. Conversely, β-catenin activation inhibited MC2R expression and corticosterone production (Walczak et al., 2014). These studies were further supported by MRAP-deficient mice, the adrenals of which had a diminished zF and an enhanced zG marked by β-catenin and LEF1 (Novoselova et al., 2018). Together, these data suggest that while high Wnt/β-catenin activity promotes a zG-like phenotype by inhibiting zF-specific gene expression, it is then inhibited along a centripetal gradient as PKA signaling is activated and zG cells concomitantly differentiate into cells of the zF.

In addition to Wnt/β-catenin and other paracrine signaling mechanisms, transitioning zG cells have been hypothesized to undergo epigenetic modifications, which play an essential role in the differentiation of multi-/pluripotent stem/progenitor cells of various organs (Margueron and Reinberg, 2011). Supporting this hypothesis is the recent finding that enhancer of zeste homolog 2 (EZH2), a histone methyltransferase involved in polycomb repressive complex 2 (PRC2)-dependent transcription repression, was widely overexpressed in an ACC patient cohort, suggesting a critical regulatory role in normal adrenal homeostasis (Drelon et al., 2016b). Work by Mathieu et al., described an *Ezh2* KO mouse model which displayed reduced adrenal size, corticosteroid insufficiency, and marked disruption of zF differentiation (Mathieu et al., 2018). Perhaps most striking was the reduction of PKA-dependent gene expression, suggesting that EZH2 is necessary for PKA activation in steroid-producing cells. These data further complexify Wnt/β-catenin-mediated transition of cellular identity between zG and zF. It was concluded that EZH2 regulates zG-to-zF transdifferentiation through PKA-dependent Wnt/β-catenin inhibition (Mathieu et al., 2018). In light of the common dysregulation of Wnt/β-catenin signaling and EZH2 in ACC, it is intriguing to speculate whether EZH2 facilitates the silencing of β-catenin-dependent transcriptional programming and/or the activity of ZNRF3 in cells transdifferentiating from zG to zF (Drelon et al., 2016b; Pignatti et al., 2020).

1.5.4 β-catenin-mediated adherens junctions and zG architecture

Target gene activation by nuclear β-catenin, as discussed so far, must be tightly regulated to maintain proper homeostasis and zonation of the adrenal cortex. However, the scope of Wnt/β-catenin signaling reaches far beyond gene regulation and plays a

crucial role in cell adhesion and tissue organization. β-catenin is an essential component of cadherin complexes that act to maintain cell-cell adhesion and reorganize intracellular actin filaments upon tissue reorganization (Aberle et al., 1994; Jou et al., 1995; Vasioukhin et al., 2000). Stabilized cytoplasmic β-catenin interacts with E-cadherin in epithelial tissues, for example, and binds to its ortholog α-catenin, linking the cadherin-catenin complex to the actin cytoskeleton to induce morphological changes (Buckley et al., 2014). The focal point of these critical cell-cell interactions is the adherens junction (AJ), the rendezvous of extracellular cadherin domains and extracellular matrix (ECM) components of multiple cells organized to maintain the structural integrity of the tissue.

The adrenal zona glomerulosa was named such in light of its composition of dense cell clusters called glomeruli (Zelander, 1957; Cater and Lever, 1954). It was further shown that these glomeruli are heterogeneous bundles of SHH-expressing/CYP11B2-negative and CYP11B2-expressing/SHH-negative cells, whereas virtually all are marked by high β-catenin activity (Walczak et al., 2014; Basham et al., 2019). Recent work from Leng et al., has provided crucial insight into the molecular and cellular characteristics of these glomeruli and the role of β-catenin in mediating zG morphology. Glomeruli of the zG were found to be surrounded by laminin β1, a basement membrane marker that clearly outlines each glomerulus (Leng et al., 2020). Furthermore, β-catenin was observed in punctate clusters at the center of the glomerulus along with F-actin and N- and K-cadherin. These results suggested to the authors the co-identity of glomeruli with rosettes—round, multicellular structures that are prominent during embryogenesis of many organs but generally disappear by adulthood. Rosettes are characterized by cadherin and β-catenin interactions bundled in the center AJ that physically links all the

clustered cells together. Evidence was provided for the postnatal development of rosettes in the adrenal zG by 6 weeks of age in mice.

Because β-catenin is a critical regulator of adrenocortical zonation, it was hypothesized that its activity was essential for proper AJ structure and, subsequently, zG morphology. Indeed, β-catenin GOF and LOF experiments resulted in an overall increase in zG thickness and rosette frequency and a thinner zG with rosette disorganization, respectively (Leng et al., 2020). Further experiments identified downstream target genes *Fgfr2IIIb* and *Shroom3* as critical regulators of AJ maintenance in the adrenal zG (Leng et al., 2020). Additional independent studies characterized rosettes as crucial components of zG-specific AngII-induced Ca^{2+} signaling, a significant contributor to both aldosterone production and extracellular AJ biology (Guagliardo et al., 2020). These studies provide a greater understanding of the Wnt/β-catenin-dependent mechanisms that govern extracellular zG identity and morphology. Wnt/β-catenin activity is central to activating downstream target genes in response to both WNT ligands and cell-cell adhesion signaling components. How this dual role of β-catenin is itself regulated in various contexts is an important question to consider. First, while β-catenin-mediated AJs are important for proper zG maintenance, their role in zG-to-zF cell transdifferentiation remains unknown (Leng et al., 2020; Mathieu et al., 2018). Since constitutive β-catenin activity was recently shown to block this transdifferentiation, leading to an expanded zG, it may be predicted that β-catenin inhibition is necessary for cellular delamination and centripetal movement out of the zG (Pignatti et al., 2020; Berthon et al., 2010). Cellular markers of nascent delaminated zG cells would provide further insight into this transient cell population predicted to be at least in part regulated by ZNRF3, PKA, and EZH2

(Basham et al., 2019; Drelon et al., 2016a; Mathieu et al., 2018). Finally, these studies beg the question if the zG is a progenitor cell compartment at least in part due to its structural morphology (King et al., 2009; Freedman et al., 2013; Walczak et al., 2014). Rosette structures, particularly in the brain, have been proposed to be stem/progenitor cell niches that maintain the unique environment necessary for multipotent cells to undergo long-term self-renewal (Fuentealba et al., 2012). In addition to the various paracrine signaling components that have been shown to regulate SHH-expressing adrenocortical progenitor cells, how β-catenin-regulated rosette architecture in the zG additionally maintains the progenitor cell niche must be further explored.

1.5.5 Interplay between endocrine signaling and β-catenin activity

Cells of the adrenal cortex are regulated by many paracrine signaling components, such as WNT4, RSPO3, ZNRF3, and SHH, to maintain both structural and functional integrity of the tissue (Heikkilä et al., 2002; Vidal et al., 2016; Basham et al., 2019; King et al., 2009). In addition to these paracrine mechanisms, major endocrine pathways, namely the renin-angiotensin-aldosterone system (RAAS) and the HPA axis, must also be encumbered in any model of adrenocortical homeostasis. Endocrine signals have been shown not only to regulate steroid hormone production in the adrenal cortex through physiological negative feedback loops, but also to induce cortical cell proliferation and regulate the thickness of concentric cortical zones (Nishimoto et al., 2014; McEwan et al., 1999; Tian et al., 1995). Low intravascular volume induces the RAAS and AngII-mediated activation of differentiated cells of the adrenal zG to proliferate and produce aldosterone, which is activated by β-catenin and WNT4 (Heikkilä et al., 2002; Berthon et al., 2010; Pignatti et al., 2020). However, the delicate interplay between endocrine AngII and

paracrine Wnt/β-catenin signaling in maintaining appropriate progenitor cell numbers and aldosterone production in the RAAS is not completely understood.

Another important consideration in Wnt/β-catenin-regulated adrenocortical zonation is the role of ACTH in promoting zG-to-zF transdifferentiation through PKA signaling activation. While the role of cAMP/PKA signaling in inhibiting β-catenin and promoting a zF phenotype in cells has already been discussed, whether ACTH only promotes corticosteroid production in existing zF cells or is also actively engaged in the transition of a zG cell to an ACTH-responsive zF cell is unknown (Drelon et al., 2016a; Mathieu et al., 2018). Moreover, it is unclear if the ACTH-dependent proliferation observed during adrenal regeneration occurs in residual zF cells or in transdifferentiating SHH-expressing or CYP11B2-expressing cells of the zG (Finco et al., 2018).

1.6 Wnt/β-catenin signaling in adrenal development

In addition to Wnt/β-catenin signaling being an important regulator of homeostasis of the adult adrenal cortex, it is also critical for the establishment and zonation of the nascent adult cortex. Adrenocortical development begins early in embryogenesis when *Sf1* expression defines the adrenogonadal primordium (AGP), which delaminates from the coelomic epithelium at E9.0 (Hatano et al., 1996; Zubair et al., 2008). Cells of the AGP further differentiate, and by E10.5 in the mouse, cells expressing higher levels of *Sf1* under the control of the fetal adrenal enhancer (*FAdE*) branch off from the developing gonads to become the fetal adrenal anlagen (Zubair et al., 2006). The medulla is then formed at E12.5 by neural crest cells invading and coalescing within the developing cortex, and the developing adrenal is fully encapsulated by mesenchymal cells of the

intermediate mesoderm at E13.5. *Ctnnb1* expression has been observed by ISH around E12.5 in the peripheral cortex, but it is still unclear how it contributes to the establishment of the definitive progenitor cell niche (Kim, A.C., et al., 2008).

Several Wnt/β-catenin signaling components contribute significantly to the growth of the definitive cortex during prenatal development in mice. Formation of the adult cortex was drastically attenuated when β-catenin was conditionally deleted using an SF1-Cre driver (Kim, A.C. et al., 2008). Additionally, the loss of capsular *Rspo3* at encapsulation (E13.5) initiated by GLI1-CreERT2 resulted in zG loss by E18.5 (Vidal et al., 2016). The use of ubiquitous CAG-CreERT-activated *Rspo3* deletion at E11.5 prior to encapsulation exacerbated this phenotype by the same end point. These data suggest capsular signals begin activating cortical Wnt/β-catenin signaling almost immediately upon encapsulation, providing essential molecular cues for definitive zonation of the adult cortex. While studies on the loss of β-catenin activation in the developing adrenal cortex have resulted in zonation defects and progenitor cell loss, mouse models with overactivation of β-catenin, such as constitutively activated β-catenin (ΔCat) and *Znrf3* KO mice, have not reported developmental defects, although collectively the data would support an early phenotype due to the necessity of β-catenin regulation for normal development (Berthon et al., 2010; Basham et al., 2019). Future studies are expected to shed light on the role of Wnt/β-catenin in the early establishment of the progenitor cell niche during specification of the adult adrenal cortex.

1.7 Wnt/β-catenin signaling in adrenal regeneration

The adrenal cortex regularly undergoes rapid molecular, cellular, and morphological changes in response to endocrine stimuli and paracrine signals between the capsule and cortex, including a Shh-Wnt signaling relay, that together regulate the cortical progenitor cell pool to establish and maintain adrenocortical zonation. Adrenal regeneration experiments have provided insights into homeostatic signaling pathways that both maintain adrenocortical zonation and mediate cell fate decisions. Using three different markers of cell populations in the zG, lineage tracing experiments have revealed that WNT-responsive (*Axin2-CreERT2*; *RGFP*) cells, undifferentiated *Shh*-expressing progenitors (*Shh-CreERT2*; *mTmG*), and differentiated *Cyp11b2* (*AS*)-expressing (*AS-Cre*; *mTmG*) cells all contribute to cortical cell renewal throughout life (Walczak et al., 2014; Finco et al., 2018; King et al., 2009; Freedman et al., 2013). Finco et al., demonstrated the importance of paracrine Shh and Wnt/β-catenin signaling pathways between the capsule and cortex, respectively, in mediating adrenocortical regeneration. Using dexamethasone therapy to suppress ACTH levels and induce zF atrophy in mice, a concomitant robust increase in *Shh* expression and activation of Wnt/β-catenin signaling in the zG was observed during regeneration after dexamethasone treatment ceased (Finco et al., 2018). Expectedly, cortical recovery was stunted by both genetic ablation of β-catenin in WNT-responsive cortical cells (*Axin2-CreERT2*; *Ctnnb1*$^{fl/fl}$) and pharmacological inhibition of capsular Shh signaling activation. Moreover, constitutively activated Shh signaling in the capsule through genetic manipulation of the SMO receptor led to an increase in Wnt/β-catenin signaling and rate of recovery, suggesting that SHH

activation of capsular GLI1-expressing cells accentuates cortical Wnt/β-catenin signaling, which in turn either directly or indirectly upregulates *Shh* expression in the zG.

The interplay between Wnt/β-catenin and Shh signaling pathways in adrenal regeneration further support the importance of the adrenal capsule in maintenance of cortical homeostasis. Interestingly, *Rspo3* has been implicated as a direct GLI1 target gene, although this has not yet been verified in the adrenal capsule (Lewandowski et al., 2015). It is also unknown whether GLI1 activates WNT ligand expression in the capsule (Lako et al., 1998; Lin et al., 2001; Pietila et al., 2016; Basham et al., 2019). Furthermore, although *Shh* is downregulated in *Rspo3* KO mice, it is unknown whether *Shh* is a *bona fide* β-catenin target gene (Vidal et al., 2016). Finally, while all zG cells are WNT-responsive, how Wnt/β-catenin signaling mediates the fate of both differentiated and undifferentiated cell populations and their contributions to homeostatic renewal of the adrenal cortex, especially under endocrine system manipulations as performed in regeneration experiments, will continue to be a challenging yet exciting avenue of research in adrenal biology. Future studies employing ChIP and single cell RNA sequencing (scRNAseq), among other techniques, will help define the underlying mechanisms of the adrenocortical Shh-Wnt relay that maintains zG identity and cortical zonation over time.

1.8 Wnt/β-catenin signaling in adrenal disease

Homeostatic molecular and cellular mechanisms of the adrenal cortex must be tightly regulated throughout life as dysregulated processes lead to a variety of disorders. Adrenal diseases range from hormonal hypo- and hyperfunction to hypo- and hyperplasia,

together with benign and malignant tumors of the cortex and medulla. Pathophysiological excess of cortisol causing Cushing syndrome, affecting thousands of patients every year, can present as familial or sporadic disease in the setting of adrenocortical hyperplasia or adrenocortical tumors (ACTs), including benign adrenocortical adenoma (ACA) and carcinoma (ACC). Molecular mediators of ACTs have recently been characterized. ACA is a common benign tumor of the adrenal cortex whereas ACC is a rare, highly metastatic malignancy with poor overall survival (reviewed in Else et al., 2014). Many adult ACCs harbor somatic inactivating mutations in *TP53* and overexpression of *TERT*, whereas the majority of pediatric ACC cases are driven by germline *TP53* mutations associated with Li-Fraumeni syndrome (Tissier et al., 2005; Else et al., 2014; Svahn et al., 2018; Ribeiro et al., 2001; Wasserman et al., 2015; Pinto et al., 2015). Tumor microarray data also defined *IGF2* overexpression in more than 90% of ACCs but not ACAs (Giordano et al., 2003; Giordano et al., 2009). On the other hand, active nuclear and cytoplasmic β-catenin has been observed in both sporadic ACA and ACC, consistent with previous observations in FAP patients harboring inactivating alterations of *APC* (Heaton et al., 2012; Smith et al., 2000; Marchesa et al., 1997). Alterations in Wnt/β-catenin signaling components have since been widely observed in aldosterone-producing adenoma (APA) of the adrenal cortex, the leading cause of primary aldosteronism (PA), as well as ACC (Conn, 1955; Fagugli et al., 2011).

Pan-genomic data from the Cochin-COMETE, European Network for the Study of Adrenal Tumors (ENSAT), The Cancer Genome Atlas ACC (TCGA-ACC) projects have recently detailed the genetic, epigenetic, and chromosomal landscape in adult ACC patient cohorts and uncovered alterations in various Wnt signaling components, such as

CTNNB1, *APC*, and *ZNRF3* (Gaujoux et al., 2011; Assié et al., 2014; Zheng et al., 2016). The following sections will outline the contributions of each Wnt/β-catenin signaling component to adrenal pathophysiology, including what is known in both mouse models and phenotypes presented in the human diseases discussed above, as well as new and ongoing studies to target this pathway therapeutically.

1.8.1 Apc

As mentioned previously, the first correlation between activated Wnt/β-catenin signaling and adrenal disease was described in patients with familial adenomatous polyposis (FAP), a hereditary disease mainly affecting the colon that is caused by germline *APC* mutations (Devic and Bussy, 1912; Smith et al., 2000; Gaujoux et al., 2010). *APC* is a tumor suppressor gene that is critical for inhibition of β-catenin by the destruction complex (Amit et al., 2002; Lee et al., 2003; Xing et al., 2004;). Inactivating alterations in both *APC* alleles, effectively nullifying negative regulation of β-catenin by phosphorylation and rendering it constitutively active, are observed in 70% of colorectal cancers (CRCs) (Rowan et al., 2000; Schell et al., 2016). In addition to presenting with several colon polyps early in life followed by a high incidence of colorectal cancer within 30 years of age, FAP patients are also at a higher risk for several other tumor types, including ACTs. This was the first evidence that β-catenin played a critical role in adrenal biology and that perturbations in the Wnt/β-catenin pathway might contribute to adrenal cancer. Later studies showed that *Apc* mutant (*Apc*^{min}) mice, which recapitulate the *APC* loss in human patients and the resulting development of colon polyps and cancer, developed adrenocortical hyperplasia and tumorigenesis later in life that was exacerbated by *Igf2* overexpression (Heaton et al., 2012; Guillaud-Bataille et al., 2014).

Interestingly, it was not until much later that the Cochin-COMETE, ENSAT, and TCGA-ACC studies all revealed a low percentage of somatic *APC* alterations in ACC patients (Gaujoux et al., 2010; Gaujoux et al., 2011; Assié et al., 2014; Zheng et al., 2016). Only about 1-3% of these patients harbored a deactivating mutation in *APC* while nearly 40% of ACCs overall had Wnt/β-catenin pathway alterations. Together with studies in *Apc*^{min} mice, these data suggested that while FAP patients harboring a germline APC mutation have increased incidence of ACC, alterations in *APC* itself are not highly prevalent in primary ACC. This highlights an interesting difference between adrenal and colon pathophysiology. Whereas *APC* loss is present in only a small fraction of ACC cases, it drives disease in nearly 70% of sporadic CRCs (Rowan et al., 2000; Schell et al., 2016). Although the same pathway is highly activated in both cancers, the tissue-specific tumorigenic potential of particular signaling component alterations has provided clues for developing unique targeted therapies through future studies.

1.8.2 β-catenin

β-catenin is the main effector of Wnt signaling and is either indirectly (as in the case of *APC* loss) or directly activated in many adrenal disease cases. Soon after the implementation of mouse models to understand the underlying effects of Wnt/β-catenin dysregulations in adrenal disease, constitutive Wnt/β-catenin signaling activation marked by diffuse cytoplasmic and nuclear β-catenin staining was found in 70% of an APA patient cohort (Kim, A.C. et al., 2008; Berthon et al., 2010; Berthon et al., 2013). While no β-catenin mutations were found in any Wnt-active APAs, *SFRP2*, an extracellular Wnt/β-catenin signaling inhibitor, was downregulated in most samples (Berthon et al., 2014). Indeed, *Sfrp2* KO mice exhibited constitutive adrenocortical Wnt/β-catenin signaling and

upregulation of *Cyp11b2*. This work demonstrated that aberrant Wnt/β-catenin signaling activation associated with APA may be caused by the downregulation of Wnt/β-catenin signaling inhibitors, such as *SFRP*2, upstream of β-catenin.

In addition to β-catenin activation by downregulation or deletion of Wnt signaling inhibitors, β-catenin itself can harbor activating mutations. *CTNNB1*, the gene encoding β-catenin, is comprised of 16 exons. The N-terminal exon 3 of *CTNNB1* contains serine and threonine (Ser/Thr) residues that are phosphorylated by the destruction complex (Liu et al., 2002; Gao et al., 2002). β-catenin regulation by phosphorylation is a critical event that must be tightly regulated in development (Amit et al., 2002). Frequent hotspot mutations at these Ser/Thr residues of exon 3 render β-catenin constitutively active and are present in a large percentage of endometrial, pancreatic, and hepatocellular cancers (Harada et al., 1999; Machin et al., 2002; reviewed in Kim and Jeong, 2019). These mutations were then discovered in a cohort of 39 ACA and ACC patients (Tissier et al., 2005). Among several SNPs in exon 3 of *CTNNB1* were those located at serine 45 (S45), which were observed in 7 or 39 (18%) ACTs as well as the human ACC NCI-H295R cell line. These mutations were verified in three large independent ACC cohorts, present in approximately 16% of cases, and correlated with poor survival (Gaujoux et al., 2011; Assié et al., 2014; Zheng et al., 2016). Interestingly, while β-catenin mutations are present in both adrenal adenomas and carcinomas, each tumor type harbors unique β-catenin-dependent transcriptional programs (Giordano et al., 2003). Future efforts are expected to define additional associated passenger mutations that contribute to β-catenin-mediated pathology.

Based on these early observations in FAP and ACT patients, mouse models harboring LOF or GOF β-catenin alterations were implemented to study the underlying role of β-catenin in normal and pathophysiologic adrenocortical biology. Whereas loss of β-catenin resulted in zonal disruption and eventual adrenal failure, constitutively active β-catenin resulting from adrenocortical AKR1B7-Cre-driven floxed exon 3 of *Ctnnb1* (ΔCat) caused adrenocortical hyperplasia and late-stage tumorigenesis, although a low percentage of mice developed ACC (Kim, A.C. et al., 2008; Berthon et al., 2010). More recent studies have provided deeper insight into the consequences of constitutive β-catenin activation in the adrenal cortex. $AS^{Cre/Cre}$ mice were used to simultaneously delete exon 3 of *Ctnnb1* and activate the RAAS, which exacerbated zG-specific hyperplasia likely due to disrupted homeostatic zG-to-zF transdifferentiation (Berthon et al., 2010; Pignatti et al., 2020). While enhanced proliferation of differentiated (aldosterone-producing) cells was observed in this context, it remains unclear whether undifferentiated progenitor cells and/or differentiated cells of the adrenal zG serve as cells of origin for β-catenin-dependent adrenocortical tumorigenesis.

Sporadic tumors of the adrenal cortex, like many other tissues, often harbor several driver and passenger mutations that are needed for advanced malignancy. While β-catenin mutations were present in about 16% of ACC patients in three separate cohorts, they widely overlapped with *TP53* loss and overexpression of *TERT* and *IGF2* (Gaujoux et al., 2011; Assié et al., 2014; Zheng et al., 2016). Importantly, adult and pediatric Li-Fraumeni patients harboring germline heterozygosity of *TP53* have an increased incidence of adrenal tumors, similar to FAP patients with *APC* loss (Li and Fraumeni, 1969a; Li and Fraumeni, 1969b; Li et al., 1988; Nichols et al., 2001; Raymond et al.,

2013). Furthermore, it had previously been hypothesized that secondary mutations were needed in ΔCat mice to advance to metastatic ACC (Berthon et al., 2010). Indeed, human patients in one cohort with either Wnt/β-catenin activation or *TP53* loss had a higher tumor-free survival rate than those with alterations in both pathways, and *p53* loss in mice enhanced the metastatic potential of ACC in ΔCat mice (Batisse-Lignier et al., 2017; Borges et al., 2020).

In vivo models of β-catenin-dependent adrenal disease, particularly ACC, have proven useful in recapitulating human adrenal diseases and determining their unique characteristics and underlying mechanisms. They have provided a foundation for new and improved preclinical models for β-catenin inhibition in ACC treatment. Drugs targeting either β-catenin itself or its many nuclear binding partners, such as CREB-binding protein (CBP), are now being widely studied in basic research and clinical trials for several cancers (Takemaru and Moon, 2000; Mullighan et al., 2011; Yu et al., 2017a). PKF-115-584 and many other compounds targeting the interaction between β-catenin and TCF/LEF coactivators have also been developed (Lepourcelet et al., 2004; Park et al., 2005; Chen et al., 2009). Treatment of the β-catenin-driven NCI-H295R ACC cell line with PKF-115-184 significantly induced cell death, supporting β-catenin as a potential therapeutic target in ACC (Doghman et al., 2008). Direct β-catenin antagonist BC2059 has shown efficacy against acute myeloid leukemia in mice (Fiskus et al., 2015). PRI-724, which inhibits the β-catenin/CBP interaction necessary for target gene activation, is currently being assessed in various Phase I and II clinical trials (Kimura et al., 2017; reviewed in Lenz and Kahn, 2014). Further developments of PRI-724-associated compounds ICG-001 and C-82 have also been undertaken to treat fibrosis and

endometrial and colorectal cancers (Emami et al., 2004; Tokunaga et al., 2017; Okazaki et al., 2019; Hirakawa et al., 2019). These studies ultimately hope to inhibit the downstream effects of constitutively active β-catenin by either blocking β-catenin from binding to target gene promoters or by inhibiting crucial target gene products to alleviate tumorigenesis in patients.

1.8.3 Znrf3

ZNRF3 is a critical negative regulator of Wnt/β-catenin signaling that was found to be deleted in about 20% of ACC patients (Assié et al., 2014; Zheng et al., 2016). The ENSAT and TCGA studies were the first to show the loss of both *ZNRF3* alleles in ACC, leading to the development of *Znrf3* KO mouse models to determine its underlying biological role in ACC. Indeed, *Znrf3* loss in mice resulted in remarkable adrenal enlargement caused by excessive proliferation of Wnt-moderate cells of the upper zF (Basham et al., 2019). It is worthy to note that, while alterations in *CTNNB1* and *ZNRF3* make up 36% of the TCGA-ACC patient cohort, these two alterations are mutually exclusive. Furthermore, *Rnf43* loss does not result in an adrenal phenotype in the mouse and so is not predicted to play a significant role in ACC (Basham et al., 2019).

The lack of overlap of *CTNNB1* and *ZNRF3* alterations is suspected as alterations in both may prove lethal for a tumorigenic cell. However, these results further highlight the importance of defining the cell of origin in ACC, which may vary in different mutational profiles. Supporting this is the result that *Znrf3* loss in mice caused expansion of Wnt-moderate cells of the upper zF, whereas tumorigenesis in ΔCat mice likely originated in the zG (Basham et al., 2019; Berthon et al., 2010; Pignatti et al., 2020). Although these observations may seemingly answer the cell of origin question, the etiology of each

alteration must continue to be studied in the small but ever-growing ACC patient dataset. Additionally, whereas patients harboring *CTNNB1* mutations may benefit from downstream inhibitors, *ZNRF3*-deficient tumors can be treated upstream of the pathway as they are likely highly sensitive to WNT and RSPO ligands, potentially even being dependent on these ligands to grow, transform, and metastasize. Clinical trials in a variety of cancers implementing inhibitors of PORCN and Wntless, cytoplasmic proteins necessary for WNT ligand post-translational modifications and secretion, are currently ongoing. Additionally, neutralizing antibodies of FZD and LGR receptors may prove effective in inhibiting β-catenin activation by WNT and RSPO ligands, respectively.

1.8.4 WNT ligands

WNT4 is the main canonical WNT ligand expressed in the adrenal cortex (Heikkilä et al., 2002). In humans, *WNT4* loss results in sex reversion, kidneys, adrenal, and lung dysgenesis (SERKAL) syndrome (Mandel et al., 2008). SERKAL syndrome is an autosomal recessive condition resulting in female to male sex reversion as well as defects in adrenal growth and function. This was modeled in female *Wnt4* KO mice in which sex reversion was observed (Heikkilä et al., 2002). In addition, a functional but not structural adrenocortical defect that resulted in decreased aldosterone levels at 12 weeks of age was also noted in *Wnt4* KO mice. It may be expected that *Wnt4* overexpression would result in adrenocortical hyperplasia, but this model has not yet been generated. It is important to note that, while WNT ligands are upregulated in many cancers, they are rarely mutated themselves. For example, in breast and GI cancers, various WNT ligands are upregulated and associated with poor survival (reviewed in Zhan et al., 2017). This holds true in ACC, in which *WNT4* is highly upregulated as a Wnt/β-catenin target gene

itself in Wnt-active tumors (Zheng et al., 2016). WNT4 may thus play a critical role in ACC, especially WNT ligand-dependent *ZNRF3*-null cases. Studies in mice harboring combined loss of *Wnt4* and *Znrf3* and other Wnt signaling components would provide deeper insight into the importance of WNT4 and perhaps other WNT ligands in activating Wnt/β-catenin signaling in ACC.

1.8.5 Frizzled/Lrp receptors

WNT ligands bind to FZD and LRP receptors to activate intracellular signaling (Adler, 1992; Cadigan et al., 1998; Pinson et al., 2000). Ten FZD (1-10) and two LRP (5 and 6) receptors are expressed in mice and humans, several of which influence an array of human diseases. LRP5/6 have been implicated in human familial exudative vitreoretinopathy (FEVR) and bone loss and in various bone and Wnt-related developmental defects in mice (Toomes et al., 2004; Gong et al., 2001; Kato et al., 2002; Pinson et al., 2000). Interestingly, one somatic *LRP6* mutation in ACC was noted (Zheng et al., 2016). *FZD4* mutations are similarly found in human FEVR (Robitaille et al., 2002). Furthermore, various phenotypes due to FZD loss have been observed in mice, affecting bone, kidney, B cell, and brain development (Albers et al., 2011; Heilman et al., 2013; Ye et al., 2011; Ranheim et al., 2005; Stuebner et al., 2010). Redundancy of many FZD receptors has also been reported, as the loss of two FZDs of a distinct subfamily results in similar phenotypes in mice (Yu et al., 2012). Some pairs, such as FZDs 3 and 6, function to elicit β-catenin-independent Wnt/PCP signaling (Wang et al., 2006). Conversely, FZDs 4 and 8 redundantly activate canonical Wnt/β-catenin signaling in the developing kidney (Ye et al., 2009). The importance of FZD receptors in the mouse adrenal has not yet been studied. However, it can be hypothesized that FZD loss would

result in a dramatic phenotype in the adrenal cortex and thus be therapeutically targetable in WNT ligand-dependent ACTs. FZD inhibition was first studied with the use of niclosamide, an anti-helminthic drug used for tapeworm infection, which blocked activation of Wnt/β-catenin signaling in vitro (Chen et al., 2009). Separate studies then led to the development of both a peptide and an antibody targeting FZD receptors (Nile et al., 2018; Gurney et al., 2012). The latter therapy, OMP-18R5, or vantictumab, targets FZDs 1, 2, 5, 7, and 8 (Gurney et al., 2012). OMP-18R5, along with the FZD8 decoy receptor OMP-54F28. Both molecules are currently in Phase I clinical trials to assess efficacy against primary and metastatic pancreatic, breast, liver, and ovarian cancers, among others (Fischer et al., 2017; Jimeno et al., 2017). A Phase Ib clinical trial for vantictumab treatment of metastatic pancreatic cancer was stopped early due to excessive bone toxicities, but these effects seem to be reversible and manageable (Fischer et al., 2017; Smith et al., 2013). FZD inhibition would provide a viable treatment option for the subset of ACC patients with tumors harboring alterations upstream of β-catenin, such as *ZNRF3* loss. While the adverse effects of FZD inhibition are significant, studies on the efficacy of FZD and LRP inhibition in mouse models of adrenocortical diseases are nonetheless urgently needed.

1.8.6 Porcupine

Similar to FZD and LRP receptors, PORCN is essential for ligand-dependent Wnt/β-catenin signaling activation. PORCN is located on the endoplasmic reticulum where it palmitoylates WNT ligands for secretion, a process that is essential for mammalian embryonic development (Kadowaki et al., 1996; Cox et al., 2010; Biechele et al., 2011). *Porcn* loss in mice recapitulates the associated human disease focal dermal

hypoplasia (FDH), an X-linked disorder that is lethal in males and causes various limb, skin, and other developmental defects in females (Barrott et al., 2011; Biechele et al., 2011; Grzeschik et al., 2007; Wang et al., 2007). PORCN is necessary for several Wnt-active cancers that are dependent on WNT ligand secretion. The role of PORCN in ACC was modeled in *Znrf3*; *Porcn* dKO mice in which the loss of adrenocortical *Porcn* partially rescued the hyperplastic phenotype cause by *Znrf3* loss, suggesting that WNT ligands may play a significant role in inducing proliferation in *ZNRF3*-null ACC (Basham et al., 2019). Reversible PORCN inhibition by the compound IWP, a commercially available drug now widely used to deactivate Wnt/β-catenin signaling in the laboratory, was one of the first studies effectively targeting PORCN in cancer cells (Chen et al., 2009). Small molecule drugs C59 and ETC-159 were later developed and utilized in treating MMTV-*WNT1*-induced mammary tumors and colorectal cancer harboring an *RSPO1* translocation, respectively (Proffitt et al., 2013; Madan et al., 2016). Several drugs, such as LGK974 (WNT974), are now in Phase I or II clinical trials for treating melanoma, colorectal, breast, GI, pancreatic, and head and neck cancers (Liu et al., 2013; reviewed in Jung and Park, 2020). The efficacy of targeting PORCN in these Wnt-active tumors provides support for its utility as a treatment for ACCs harboring deletion of *ZNRF3* or overexpression of WNT ligands or receptors. It is therefore likely that PORCN inhibitors would pose therapeutically beneficial in such patients. Inhibition of PORCN would deplete the activating signal for Wnt-responsive cells to either manage disease burden, such as steroid hormone excess, or to effectively inhibit cancer cell proliferation, whether administered alone or in combination with other therapeutic strategies.

1.8.7 Rspo/Lgr

LGR receptors and their RSPO ligands are essential signaling components in the Wnt/β-catenin sphere. Embryonic loss of *Rspo3*, *Lgr4*, or *Lgr5* results in pre- or postnatal lethality (Aoki et al., 2007; Mazerbourg et al., 2004; Morita et al., 2004). The Wnt-potentiating RSPO/LGR signaling module has also been found to be aberrantly activated in a variety of cancers. *LGR5*, a marker of intestinal stem cells, promotes CRC cell survival in mice and was found to be overexpressed in 64% of one patient cohort (Barker et al., 2007; Al-Kharusi et al., 2013; McClanahan et al., 2006). Upregulation of *LGR5* has also been noted in several ovarian, breast, and hepatocellular cancers (McClanahan et al., 2006; Liu et al., 2018; Hou et al., 2018; Ko et al., 2019). Additionally, RSPO proteins, particularly RSPOs 2 and 3, have been implicated as potential drivers in CRC and liver and lung cancers (Starr et al., 2009; Conboy et al., 2019; Gong et al., 2015). Recurrent translocation events in CRC leading to overactive RSPO fusion proteins have also been widely observed (Seshagiri et al., 2013). However, conflicting studies have reported that overactive RSPO/LGR signaling may be therapeutically beneficial. Overexpression of *RSPO1-3* correlated with increased survival in a lung patient cohort (Wu et al., 2019). Another study reported a tumor suppressive effect of the RSPO2/LGR5 module in CRC (Wu et al., 2014). These differences highlight the crucial need for more prospective and retrospective studies to determine the underlying effects of aberrant RSPO/LGR signaling in cancer patient cohorts. Surprisingly, no mutation, amplification, or translocation events involving *RSPO1-4* or *LGR4/5/6* were reported in two ACC patient cohorts (Assié et al., 2014; Zheng et al., 2016). However, evidence suggests that inhibition of RSPO/LGR signaling could potentially dampen aberrant Wnt/β-catenin signaling activation in ACC, especially in *ZNRF3*-null tumors (Basham et al., 2019). Future basic and preclinical

studies are needed to more fully understand the role of RSPO/LGR-dependent Wnt/β-catenin signaling potentiation in ACTs.

1.8.8 Other Wnt antagonists

In addition to *APC* and *ZNRF3*, various other Wnt signaling antagonists exist to help regulate the pathway. *Axin2*, a classic β-catenin target gene that partakes in a negative feedback loop on the Wnt/β-catenin pathway, is enriched in the zG of the adrenal cortex. AXIN2 interacts with APC and GSK3β in the β-catenin destruction complex. Mutations in *AXIN1/2* affecting the destruction complex and β-catenin binding sites have been observed in a variety of cancers, including CRC (Liu et al., 2000; Webster et al., 2000). Adrenal tumors are mostly devoid of *AXIN1/2* mutations, with deletions being observed in two adenomas and one ACC in two separate cohorts (Chapman et al., 2011; Guimier et al., 2013; Assié et al., 2014). These data suggest that AXIN2 does not play a significant role in ACC development and thus may not be a strategic therapeutic target.

As mentioned, PKA activation inhibits Wnt/β-catenin signaling and drives differentiation in the upper zF (Drelon et al., 2016a; Dumontet et al., 2018). Constitutively activating somatic mutations in *PRKACA*, encoding the catalytic subunit of PKA, have been observed in nearly half of adrenal adenoma patients and are associated with bilateral adrenal hyperplasia and resulting cortisol excess (Cushing syndrome) (Zilbermint and Stratakis, 2015; Weigand et al., 2017). Adrenocortical hyperplasia and Cushing syndrome was also observed in mice lacking the regulatory subunit of PKA encoded by *Prkar1a*, mutations of which result in constitutive PKA activation and occur frequently in primary pigmented nodular adrenocortical disease (PPNAD) (Sahut-Barnola et al., 2010; Dumontet et al., 2018). Additionally, alterations in *PDE11A* and *GNAS*,

regulators of PKA signaling, have been observed in ACTH-independent macronodular adrenal hyperplasia (AIMAH) and McCune-Albright syndrome, respectively (Vezzosi et al., 2012; Weinstein et al., 1991). However, somatic mutations and deletions were observed in *PRKAR1A* but not *PRKACA* in human ACC (Assié et al., 2014; Zheng et al., 2016). Indeed, loss of *Prkar1a* induced adrenocortical tumorigenesis and corticosterone excess in mice, expected to be due to increased zF proliferation (Drelon et al., 2016a). Conversely, heterozygous loss of *Prkaca* resulted in β-catenin-induced tumorigenesis, which was partially abrogated by *Wnt4* loss. These data suggest that PKA acts as a tumor suppressor in the adrenal cortex by inactivating Wnt/β-catenin signaling in the upper zF. Based on their roles, it can be proposed that PKA activation through recurrent *PRKAR1A* deletion is mutually exclusive with activating β-catenin mutations in ACC, leading to different clinical and prognostic outcomes. Aberrant PKA signaling has been described in PPNAD patients and mouse models, whether by alterations in PKA, *GNAS*, or various phosphodiesterases (Boikos et al., 2008; Mathieu et al., 2018; Pignatti et al., 2020; reviewed in Fragoso et al., 2015). Conversely, *CTNNB1* mutations are associated with non-steroid-secreting tumors (Bonnet et al., 2011). However, further analyses must be done to understand the role of PKA signaling in human ACC.

In addition to intracellular regulators of Wnt/β-catenin signaling, secreted factors of the Dikkopf (DKK) family of proteins also negatively regulate the pathway at the cell surface by binding to LRP5/6 receptors (Glinka et al., 1998; Fedi et al., 1999). *Dkk3* inactivation was observed in a *Kcnk3* KO mouse model recapitulating human PA (El Wakil et al., 2012). Combined loss of *Kcnk3* and *Dkk3* resulted in further upregulation of *Cyp11b2*, suggesting that DKK3 acts to negatively regulate β-catenin-induced *Cyp11b2*

expression and aldosterone production. However, neither adrenocortical hyperplasia or tumorigenesis were observed, further supporting the need for alterations in crucial regulators and highlighting the complexity of Wnt/β-catenin signaling regulation in adrenocortical biology.

1.9 Summary

Wnt/β-catenin signaling governs the development and homeostasis of several tissues throughout the body. The remarkable history of Wnt signaling research has led us to a deeper understanding of organ maintenance and human diseases that occur due to aberrant pathway activation. The adrenal gland is one of many organs dependent upon Wnt/β-catenin signaling for lifelong renewal and function. Differentiated cells of the adrenal cortex rapidly produce crucial steroid hormones in response to various stressors. The peripheral zona glomerulosa (zG) contains aldosterone-producing cells and undifferentiated progenitor cells. Fate and function of both zG cell populations are guided in part by Wnt/β-catenin through communication with the overlying capsule. To maintain proper homeostatic renewal, this process is tightly regulated by antagonists like ZNRF3. Alterations of several Wnt/β-catenin signaling components drive aberrant aldosterone production and adrenocortical tumorigenesis. Recent historic advancements in adrenal research and vital developments of numerous Wnt/β-catenin inhibitors provide an exciting foundation for new preclinical models in treating Wnt-active diseases of the adrenal cortex.

CHAPTER 2 Adrenal Capsule-Derived WNT2B is Essential for zG Development, Identity, and Maintenance

2.1 Disclosure of relevant publications

Portions of this work are being prepared for publication:

> **Little III DW***, Borges KS*, Basham KJ, Azova S, O'Connell AE, Dumontet T, LaPensee CR, Breault DT, Hammer GD. WNT2B is essential for adrenal glomerulosa identity and function. In preparation. *co-first author

2.2 Introduction

Wnt signaling was first discovered in *Drosophila* in the 1970s when the first WNT ligand (*wingless*/*Wnt1*) was described (Sharma and Chopra, 1976; Nusse and Varmus, 1982; Nusse et al., 1991). The WNT ligand family has since expanded to 19 paralogous secreted glycoproteins with various roles in cell proliferation, guidance of cell polarity, maintenance of stem/progenitor cells, and differentiation by binding to transmembrane FZD receptors that initiate the intracellular Wnt/β-catenin pathway. Subsequent activation of β-catenin-dependent transcriptional programs contributes to processes ranging from organogenesis to tumorigenesis. While the importance of canonical Wnt/β-catenin signaling in the adrenal cortex has been known for decades, the precise WNTs involved have remained largely elusive. Previously, the only adrenocortical WNT described was *Wnt4*, which is expressed in the zG and is essential for proper aldosterone production (Heikkilä et al., 2002). However, this phenotype did not recapitulate that observed in β-

catenin LOF mice, which had pronounced disruption of adrenocortical zonation and late-stage failure (Kim, A.C. et al., 2008). Moreover, capsule-derived RSPO3 was defined as a critical potentiator of Wnt/β-catenin-mediated adrenocortical zonation (Vidal et al., 2016). Therefore, it was suspected that at least one capsule-derived WNT ligand was present to activate zG-restricted Wnt/β-catenin signaling. Recent work in our lab has revealed capsular expression of *Wnt2b*, a previously unknown ligand of the Wnt/β-catenin signaling pathway in the adrenal cortex. In this chapter, I will describe the known biological roles of WNT2B in tissue development and homeostasis, followed by our data highlighting its importance in adrenocortical structure and function.

Wnt2b was first cloned and characterized as *Wnt13* in mice, in which it is expressed in several developing tissues (Katoh et al., 1996). *Wnt13* was found to have high homology with *Wnt2* and thus renamed *Wnt2b* (Zakin et al., 1998). Soon after, two isoforms of *Wnt2b* were found in humans, designated *WNT2B1* and *WNT2B2*, the latter having complete identity with mouse *Wnt2b* except for the N terminal region (Katoh et al., 2000). Orthologous *Wnt2b* expression was also discovered in *Xenopus* and zebrafish, having a role in body axis patterning and organogenesis early in development (Landesman and Sokol, 1997; Landesman et al., 2002; Kunz et al., 2004; Garriock et al., 2007; Damianitsch et al., 2009; Rankin et al., 2012; Fischer et al., 2003; Ober et al., 2006; Mercader et al., 2006; Wakahara et al., 2007; Neto et al., 2012). Similar roles for *Wnt2b* in cell proliferation, cell fate determination, and stem cell maintenance were discovered in developing chick embryos (Jasoni et al., 1999; Fuhrmann et al., 2000; Kawakami et al., 2001; Fokina and Frolova 2006; Jho et al., 2002; Cho and Cepko, 2006; Müller et al.,

2007; Kubo and Nakagawa, 2009; Satoh et al., 2010; Haynes et al., 2013; Moura et al., 2014).

Several studies in mice have further exemplified the importance of *Wnt2b* in tissue development and homeostasis, including in the brain, pancreas, foregut, lung, and kidney (Shimogori et al., 2004; Heller et al., 2002; Goss et al., 2009; Steimle et al., 2018; Iglesias et al., 2007). As with many WNT ligands, the precise function of WNT2B is largely tissue- and context-dependent. For example, differentiation of mouse embryonic stem cells and human teratocarcinoma cells *in vitro* was associated with increased levels of *Wnt2b* expression (Lako et al., 2001; Wakeman et al., 1998). Conversely, WNT2B inhibited mouse retinal cell differentiation by downregulating neural differentiation genes to maintain the undifferentiated progenitor cell pool (Kubo et al., 2003; Kubo et al., 2005; Steinfeld et al., 2017). The role of WNT2B in progenitor cell maintenance and cell proliferation, processes that commonly require Wnt/β-catenin signaling, has been reported in mouse vascular, colon, and urinary tract cells as well as human hematopoietic stem cells (DiRenzo et al., 2016; In et al., 2020; Worst et al., 2017; Van Den Berg et al., 1998). *Wnt2b* expression was also significantly elevated in preterm umbilical cord-derived embryonic stem cells, which subsequently decreased with increasing gestational age, suggesting a role for WNT2B in maintaining early-stage pluripotent stem cell populations (Iwatani et al., 2017).

Wnt/β-catenin signaling is essential not only for organ development, but its developmental functions are often hijacked and altered in human diseases. Indeed, both developmental and disease studies have defined WNT2B as a canonical WNT ligand that acts through β-catenin in responsive cell populations. WNT2B activates β-catenin-

dependent gene transcription to promote retinal cell differentiation, kidney and lung development, vascular cell proliferation, and liver homeostasis (Liu et al., 2003; Iglesias et al., 2007; Goss et al., 2009; DiRenzo et al., 2016; Yuan et al., 2017). β-catenin activity is promoted by WNT2B in gastric cancer, in which it is commonly upregulated (Katoh et al., 2001; Katoh, 2001; Peng et al., 2015; Zhang et al., 2021a). Activation of Wnt/Beta-catenin signaling by WNT2B has also been shown in a myriad of other human cancers, including leukemia, osteocarcinoma, cervical cancer, and hepatocellular carcinoma (Simon et al., 2005; Khan et al., 2007; Bunaciu et al., 2008; Peng et al., 2015; Memarian et al., 2012; Dai et al., 2021; Chen et al., 2019; Liang et al., 2021; Jiang et al., 2021).

Perhaps the best characterized model of WNT2B function and that which is most pertinent to this dissertation work on the adrenal gland is that of the intestine. The majority of colorectal cancers harbor activated Wnt/β-catenin signaling, usually due to inactivating *APC* mutations (Rowan et al., 2000; Schell et al., 2016). To better understand the scope of Wnt/β-catenin signaling components in CRC, Gregorieff et al., performed gene expression analyses in adult mouse intestine. Similar to the overlying mesenchymal cells of the adrenal capsule, intestinal mesenchymal cells expressed high levels of *Wnt2b* (Gregorieff et al., 2005). These mesenchymal cells were later defined as GP38+/CD34+ mesenchymal stem cells that also produce RSPO1, a critical potentiator of Wnt/β-catenin signaling in the intestine (Stzepourginski et al., 2017). Intestinal WNT2B, along with WNT3, was later shown to bind to FZD7 (Flanagan et al., 2015). Loss of FZD7 resulted in depleted intestinal stem cell crypt structure *in vivo* and organoid growth *in vitro*, suggesting that the WNT3 and/or WNT2B interaction with FZD7 is essential for LGR5+ stem cell maintenance. The importance of WNT2B alone in this process was further

highlighted in mice lacking *Wntless*, a processing enzyme required for WNT ligand secretion (Valenta et al., 2016). Ablation of intestinal stem cell crypts in mice lacking *Wntless* was partially rescued by the addition of exogenous WNT2B alone which restored crypt structure as shown by cells marked by active β-catenin, target gene expression, and proliferation marker KI67. Intestinal injury experiments later suggested that WNT2B activates Wnt/β-catenin signaling in quiescent TERT+ intestinal stem cells to promote their rapid self-renewal for injury repair (Suh et al., 2017). Together, these data provide a foundational model for the role of mesenchymal-derived WNT2B in stem/progenitor cell maintenance through activating β-catenin signaling and cell proliferation.

Considering its essential function in intestinal homeostasis and tissue repair, it is not surprising that WNT2B also plays a fundamental role in intestinal disease. Intestinal samples from patients with ulcerative colitis (UC), a type of inflammatory bowel disease (IBD) marked by chronic colonic inflammation, had significantly elevated levels of *WNT2B* (You et al., 2008). Interestingly, hypermethylation of the *WNT2B* promoter was detected in patients with Crohn's disease (CD), a fibrotic type of IBD associated with a broader range of intestinal tissue (Sadler et al., 2016). *WNT2B* hypermethylation, resulting in its downregulation, is contrasted by separate studies showing *Wnt2b* upregulation in heart and liver fibrosis in mice, suggesting a role in tissue repair (Mizutani et al., 2016; Yuan et al., 2017). However, further studies have shown that *WNT2B* expression is upregulated in CD-associated intestinal tissue and plays a direct role in tissue repair in IBD mouse models, highlighting the complexity of Wnt/β-catenin signaling in differing disease contexts (Ortiz-Masià et al., 2020; Cosín-Roger et al., 2016). Nonetheless, *WNT2B* loss in human patients has been associated with structural and functional disorders of the eye

and intestines (Bisgaard et al., 2007; Zhang et al., 2021b; O'Connell et al., 2018). The latter study further supported an essential role for WNT2B in intestinal homeostasis as its loss results in chronic neonatal diarrhea, a phenotype that has serendipitously strengthened the connection between intestinal and adrenal biology.

Studies on the importance of Wnt/β-catenin signaling in adrenocortical structure and function have previously been highlighted (Heikkilä et al., 2002; Kim, A.C. et al., 2008; Berthon et al., 2010; Vidal et al., 2016). Given the vast body of data supporting WNT2B as a canonical WNT ligand involved in tissue development, homeostasis, and function, **we hypothesized that WNT2B expressed by the adrenal capsule is the main activator of adrenocortical β-catenin activity that subsequently modulates zG cell identity and function.** The downstream effects of WNT2B-mediated β-catenin activity were suspected to be of critical importance for proper adrenocortical development, zonation, and steroidogenesis, studies on which make up the bulk of this dissertation. In the following sections, I detail the phenotype of *WNT2B*-null patients pertinent to this thesis **(Section 2.3)** and data from our collaborative effort to define the effect of WNT2B loss on adrenocortical cell processes **(Sections 2.4-2.10)**.

2.3 *WNT2B*-null patients have elevated renin and compensated aldosterone

To investigate the role of WNT2B in human zG homeostasis, we identified three patients with established *WNT2B* deficiency and assessed their renin-angiotensin-aldosterone-system (RAAS; schematized in **Figure 2.1**) **(Table 2.1; work done by S Azova and DT Breault)**. The first two patients (A and B) came to clinical attention during the newborn period as a result of congenital diarrhea requiring fluid resuscitation and parenteral nutrition, which was attributed to homozygous loss-of-function (LOF) mutations in the *WNT2B* gene (O'Connell et al., 2018). The third patient (C) also presented with CD, as well as bilateral corneal clouding and atypical genital development (later diagnosed with 46,XX testicular disorder of sex development), which were attributed to compound heterozygous LOF mutations in the *WNT2B* gene (Zhang et al., 2021b). Analysis of RAAS activity in patients A and B at 9 and 4 years of age, respectively, revealed a normal aldosterone level but a low aldosterone-renin ratio due to markedly elevated renin concentrations **(Table 2.1)**. Analysis of patient C at 23 months of age revealed mild hyperkalemia and acidosis as well as a persistently low-normal aldosterone level, increased plasma renin activity, and low aldosterone-renin ratio (Martinez-Aguayo et al., 2010). All three patients had an intact HPA axis. Following treatment of patient C with fludrocortisone, a corticosteroid used to replace aldosterone in primary adrenal insufficiency, electrolytes and plasma renin activity normalized. Taken together, these patients with *WNT2B* deficiency show clinical evidence for subclinical hypoaldosteronism, consistent with a primary disorder of zG homeostasis in humans. Further monitoring will be required to determine whether these patients also develop glucocorticoid insufficiency.

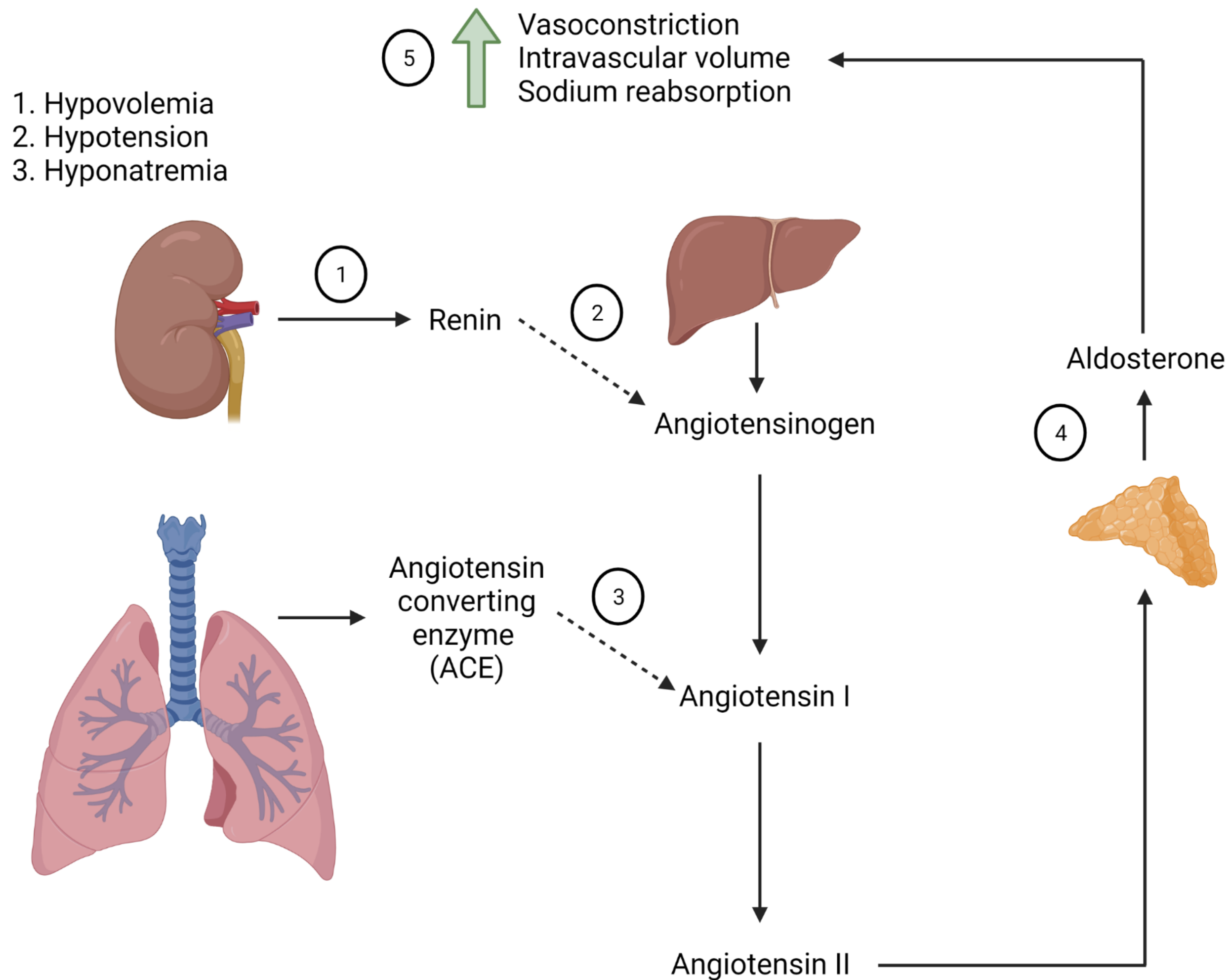


Figure 2.1. Schematic of renin-angiotensin-aldosterone system (RAAS)
(1) Hypovolemia (volume loss), hypotension, and hyponatremia (sodium loss) stimulate renin secretion from the kidneys. (2-3) Renin converts angiotensinogen secreted by the liver into angiotensin I, which is then converted to angiotensin II by angiotensin converting enzyme (ACE) produced by the lungs. (4) Angiotensin II binds directly to cells of the adrenal zG to activate *Cyp11b2* expression and subsequent aldosterone production and secretion. (5) Plasma aldosterone then acts to stimulate sodium reabsorption in the kidneys, leading to increased blood pressure and intravascular volume (IVV). Aldosterone also shuts off renin production, mediating a physiological negative feedback loop. Created with BioRender.

Table 2.1. Baseline biochemical characteristics of three patients with *WNT2B* mutations at diagnosis of subclinical hypoaldosteronism†

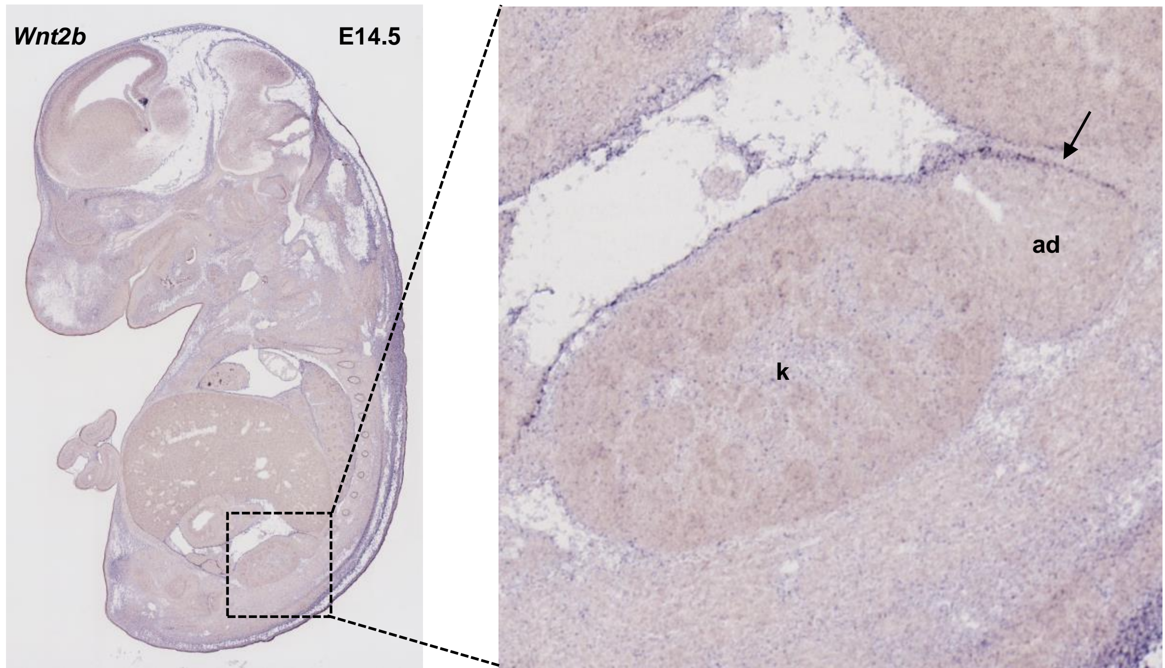
Variable **[Reference Range]**	**Patient A**	**Patient B**	**Patient C**
Sodium (mmol/L)	**134** [135-150]	136 [135-150]	136 [135-148]
Potassium (mmol/L)	3.60 [3.00-5.50]	5.00 [3.00-5.50]	**4.67** [3.20-4.50]
Chloride (mmol/L)	109 [98-110]	100 [98-110]	102 [99-111]
Bicarbonate (mmol/L)			**20** [22-32]
Aldosterone	583 [140-2220 pmol/L]	323 [140-2220 pmol/L]	12 [7-54 ng/dL]
Plasm renin concentration or activity	**286.6** [5.41-34.5 ng/L]	**341.1** [5.41-34.5 ng/L]	**18.0** [1.7-11.2 ng/mL/h]
Aldosterone-renin ratio	**2.02** [10.20-23.70 pmol/ng]	**0.93** [15.70-41.90 pmol/ng]	**0.67** [0.80-13.10]*

* The reference range for the aldosterone-renin ratio for Patient C is based on the reference values (3rd to 97th percentile) calculated by Martinez-Aguayo et al., (2010) for 211 healthy normotensive children, aged 4-16 years, with both normotensive (n = 98) and hypertensive (n = 113) parents. All other reported references ranges were provided by the respective laboratories where these variables were analyzed.

†Patient care and analysis provided by S Azova and DT Breault

2.4 *Wnt2b* is expressed in the mouse adrenal capsule

While the necessity of Wnt/β-catenin signaling in adrenal development and homeostasis has long been appreciated, the exact WNT ligands contributing to its activation had not been fully explored previously. However, a few key studies provided rationale for the expression of a WNT ligand in the adrenal capsule. The presence of *Rspo3*, a potent Wnt/β-catenin signaling potentiator, in the capsule and the substantial zG depletion caused by its loss strongly suggested the co-expression of a WNT ligand (Vidal et al., 2016). Indeed, previous work showing that *Wnt4* loss in the adrenal zG results in no structural or morphological phenotype—as observed in both *Rspo3* and β-catenin KO mice—further supported the hypothesis that cells of the adrenal capsule express a Wnt that at least in part activates cortical Wnt/β-catenin signaling (Heikkilä et al., 2002; Vidal et al., 2016; Kim, A.C. et al., 2008). To assess all WNT ligands expressed in the adrenal gland, Drelon et al. utilized GenePaint *in situ* hybridization (ISH) data, which shows expression of *Wnt2b* in the adrenal periphery at E14.5 (Drelon et al., 2015; **Figure 2.2**). A previous study on the role of *Wnt2b* in the developing kidney showed similar *Wnt2b* expression in the adrenal capsule (Lin et al., 2001). To obtain clearer resolution as to the expression patterns of *Wnt2b* and other WNT ligands in the adult mouse adrenal, we performed single molecule *in situ* hybridization (smISH) using RNAScope technology. Indeed, while several WNT ligands are expressed in the adrenal capsule and cortex, *Wnt2b* is highly enriched exclusively in the capsule **(Figure 2.2)**. **Therefore, we hypothesized that WNT2B is the key WNT ligand acting alongside RSPO3 to activate adrenocortical Wnt/β-catenin signaling and maintain zG identity.**



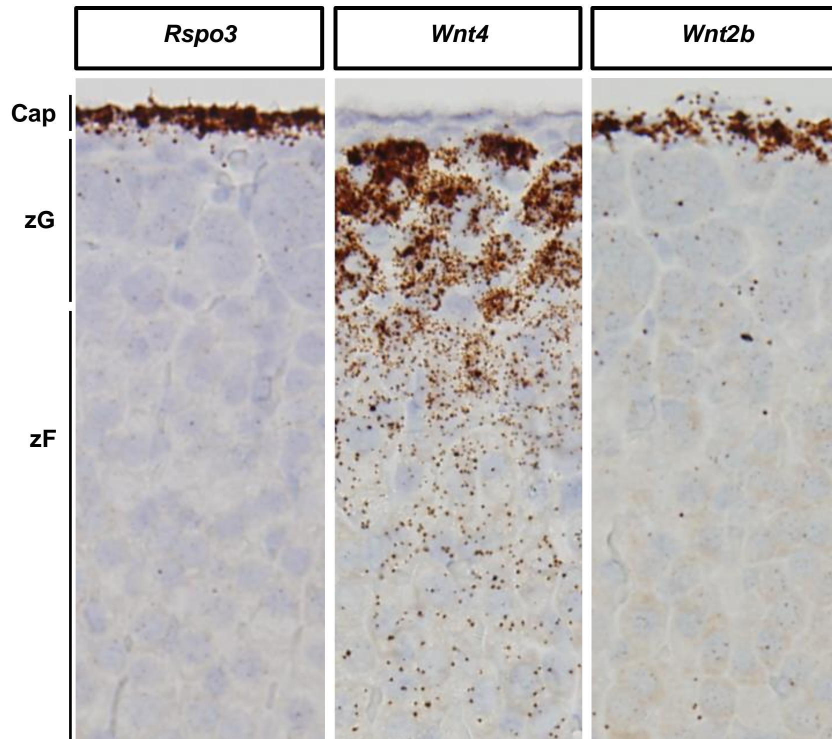


Figure 2.2. *Wnt2b* is expressed in the adrenal capsule
(Top) Expression of *Wnt2b* detected by ISH in a 14.5-day-old (E14.5) developing mouse embryo. Magnified image on right shows *Wnt2b* expression (purple) surrounding the adrenal (ad) adjacent to the kidney (k). Images obtained from GenePaint; first published in Drelon et al., 2015. (Bottom) Single molecule ISH for adrenal *Rspo3* (capsule, left), *Wnt4* (zG and upper zF, middle), and *Wnt2b* (capsule, right) in adult mice.

2.5 Characterization of the hypomorphic floxed *Wnt2b* allele

Given the new discovery of WNT2B in the adult mouse adrenal capsule and its probable role in the human RAAS, we sought to develop a mouse model to study the underlying molecular mechanisms regulated by WNT2B in the adrenal cortex. The development of a *Wnt2b* whole body KO mouse model generated from mice harboring recombined *Wnt2b*-floxed alleles provided the first opportunity to define the effect of *Wnt2b* loss *in vivo* (Tsukiyama and Yamaguchi, 2012). *Wnt2b* KO mice were viable and fertile, despite a lower number of birthed *Wnt2b* KO pups reported. Moreover, *Wnt2b* KO mice showed evidence only of a shortened olfactory bulb, consistent with *Wnt2b* expression in the developing mouse brain (Shimogori et al., 2004). Other *Wnt2b*-expressing tissues, including kidney, eye, limb, and liver, were all reported as histologically normal (Tsukiyama and Yamaguchi, 2012).

While the viability of *Wnt2b* KO mice provided a model to study the effects of WNT2B in the adult adrenal gland, we sought to utilize the *Wnt2b*-floxed line and a targeted Cre driver to allow for more detailed temporal reduction of *Wnt2b* in adult mice to study its role in adrenal homeostasis, which was not previously reported. *Wnt2b*-floxed mice were generously gifted to us for adrenal studies by Dr. Terry Yamaguchi (NCI) and were successfully validated by genotyping as reported previously (Tsukiyama and Yamaguchi, 2012) **(Figure 2.3)**.

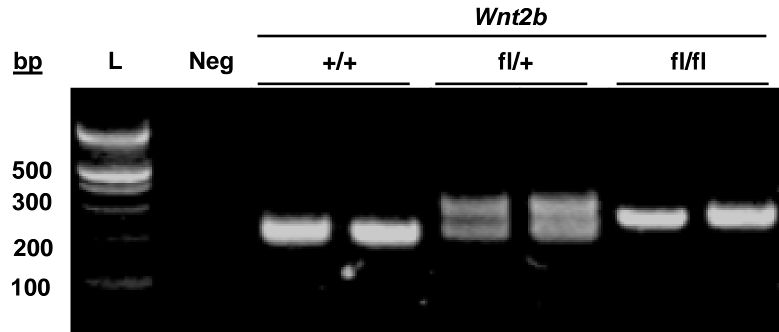


Figure 2.3. Genotyping of *Wnt2b*-floxed mice
Genotypes of *Wnt2b*-floxed mice were validated using previously reported KO1 and KO3 primers (Tsukiyama and Yamaguchi, 2012). Two representative PCR reaction samples from *Wnt2b* wild-type (+/+), heterozygous (fl/+) and homozygous (fl/fl) are shown from left to right. WT *Wnt2b* product size = 200 bp; floxed product size = 260 bp. L = ladder. Neg = negative water control.

Interestingly, upon initial analysis, decreasing levels of *Wnt2b* expression in the adrenal were found between WT, heterozygous, and homozygous *Wnt2b*-floxed mice without Cre **(Figure 2.4)**. The reduction in *Wnt2b* expression is likely due to a Neo cassette present in the floxed *Wnt2b* allele (Tsukiyama and Yamaguchi 2012). Previous studies have shown that remaining Neo cassettes can hinder normal expression patterns of affected genes (Pham et al., 1996; Revell et al., 2005; Meier et al., 2010). Since these data suggest that the floxed *Wnt2b* allele is hypomorphic, we hypothesized that the significant reduction in *Wnt2b* expression in *Wnt2b*$^{fl/+}$ and *Wnt2b*$^{fl/fl}$ mice would serve well to study its role in adrenocortical homeostasis and that the addition of a Cre driver to establish a true *Wnt2b* conditional KO (cKO) would significantly exacerbate any observed phenotype (detailed in the following sections).

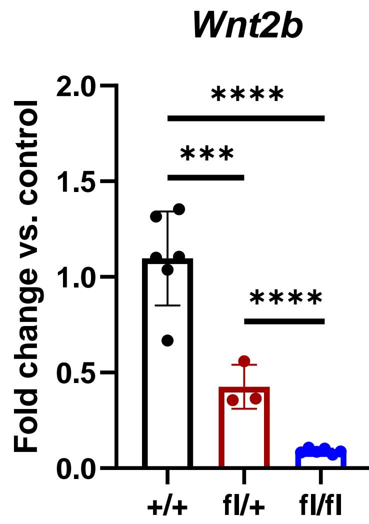


Figure 2.4. *Wnt2b*-floxed allele is hypomorphic
qPCR revealing decreasing *Wnt2b* expression in adrenals from *Wnt2b* +/+, fl/+, and fl/fl mice. Statistics performed using one-way ANOVA; ***p<0.001; ****p<0.0001.

2.6 Capsular WNT2B is necessary for zG development in mice

In light of our data from patients with *WNT2B* loss resulting in congenital subclinical hypoaldosteronism and elevated renin, we sought to define the role of WNT2B in adrenocortical development. Although whole body loss of *Wnt2b* was previously found to result in generally normal anatomy, its effect on the adrenal cortex or the RAAS was not determined (Tsukiyama and Yamaguchi, 2012). To study the role of developmental WNT2B loss, we developed a global *Wnt2b* knockout (gKO) mouse model in collaboration with the lab of Dr. David Breault (Boston Children's Hospital). Mice harboring CMV-Cre, a constitutive Cre driver present in all cells throughout the body, were crossed with *Wnt2b*-floxed mice to generate a *Wnt2b* gKO model. 6-week-old male and female *Wnt2b* gKO mice have significantly lower adrenal-to-body weight ratios than wild-type and heterozygous controls, suggesting that WNT2B plays a fundamental role in the embryological development and growth of the adrenal gland **(Figure 2.5; work done by KS Borges).**

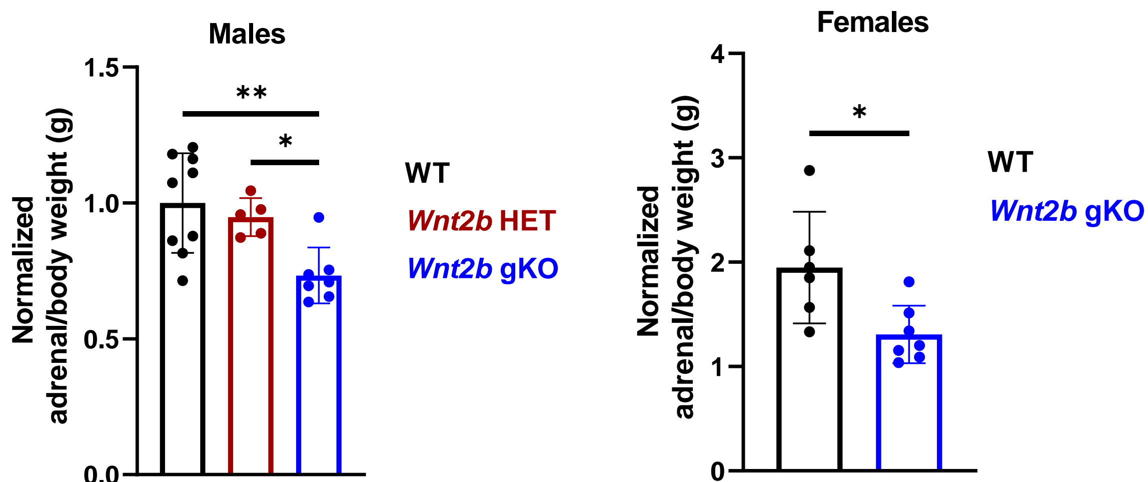


Figure 2.5. Adrenal-to-body weight ratios of *Wnt2b* gKO mice
Adrenal weights from 6-week-old male and female control and *Wnt2b*$^{fl/fl}$ mice harboring global *CMV-Cre* expression were normalized to body weight. Significant decrease is seen in *Wnt2b* gKO mice compared to +/+ (WT) and fl/+ (HET) controls. Statistics performed using one-way ANOVA (males) and Student's t test (females); *p<0.05; **p<0.01. Work done by KS Borges.

Next, to determine the effect of *Wnt2b* loss on adrenocortical zonation, which must be properly established for normal function throughout life, we performed hematoxylin and eosin (H&E) and immunofluorescent (IF) staining on *Wnt2b* WT, heterozygous, and gKO adrenals. H&E on *Wnt2b* gKO adrenals shows a lack of histological zG usually observed by dense subcapsular cell clusters. Indeed, to our great surprise, IF staining on zonal markers DAB2 (zG) and AKR1B7 (zF) reveal a complete lack of zG in *Wnt2b* gKO mice **(Figure 2.6; work done by KS Borges)**. Whereas WT mice have clear subcapsular DAB2 staining, *Wnt2b* gKO mice exhibit AKR1B7 expression all the way to the capsule. These results also suggest that *Wnt2b* loss does not affect zF development and maintenance, in line with data from *WNT2B*-null patients who have intact HPA axes. To further assess the effect of *Wnt2b* loss on adrenocortical zonation, we then analyzed Wnt/β-catenin signaling components that are highly expressed in the zG of wild-type mice. Again, both β-catenin and its target gene product LEF1 are absent from the adrenal cortex of *Wnt2b* gKO mice **(Figure 2.7; work done by KS Borges)**. Together, these data suggest that WNT2B is essential for embryological development of the adrenal zG, a critical progenitor cell niche and the body's primary source of physiological aldosterone.

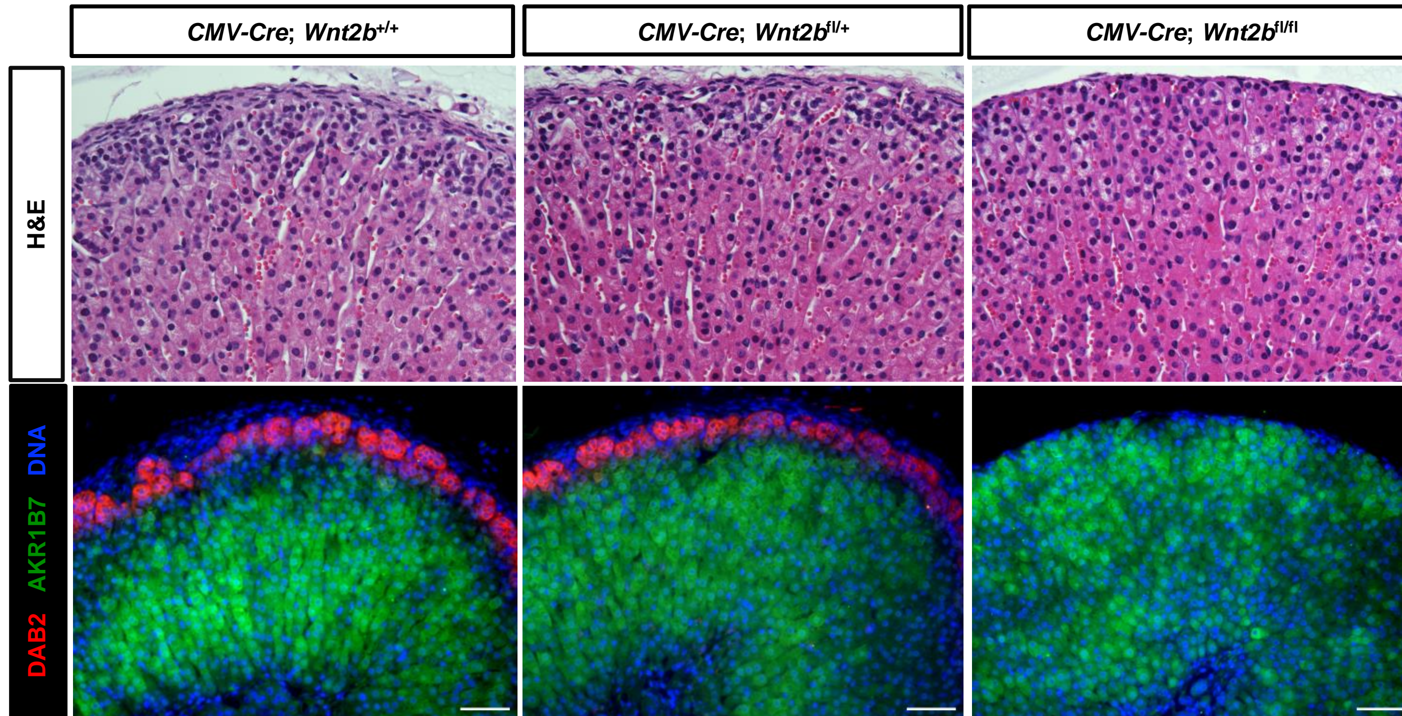


Figure 2.6. *Wnt2b* gKO mice lack histological adrenal zG
Hematoxylin and eosin (H&E; top) and immunofluorescent (IF; bottom) staining of zonation markers on adrenals from 6-week-old male *Wnt2b* +/+, fl/+, and fl/fl harboring CMV-Cre. (Top) Dense cell clusters making up the adrenocortical zG can be observed in the subcapsular regions of WT and HET adrenals. Note the lack of these zG clusters in *Wnt2b* gKO adrenals, which exhibit strands of larger, cytoplasm-rich cells of the zF to the outer cortex. (Bottom) Expression of zonal markers in the adrenal cortex. DAB2 (red) labels the zG and AKR1B7 (green) marks the zF. Proper zonation is seen in WT and HET mice, whereas *Wnt2b* gKO adrenals have no DAB2 staining, indicating zG loss. Scale bar: 100 um. Work done by KS Borges.

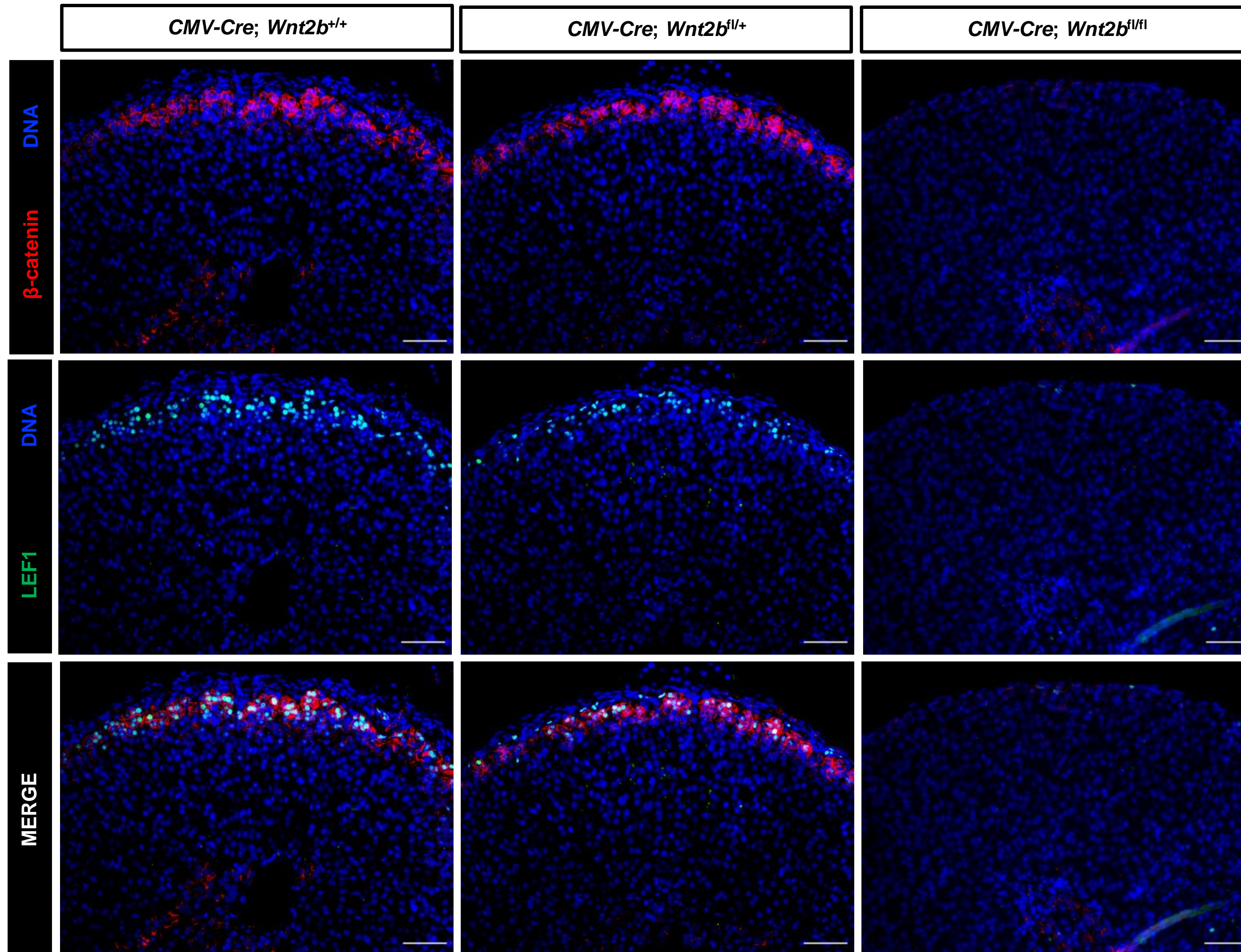


Figure 2.7. Wnt/β-catenin signaling markers are absent in *Wnt2b* gKO adrenals
IF staining for β-catenin (red) and LEF1 (green) on adrenals from 6-week-old male *Wnt2b* +/+, fl/+, and fl/fl mice harboring *CMV-Cre*. Adrenocortical β-catenin and LEF1 co-staining (bottom) can be observed in the subcapsular zG in WT (left) and HET (middle) adrenals but is absent in adrenals from *Wnt2b* gKO mice (right). Scale bar: 100 um. Work done by KS Borges.

2.7 Adrenocortical cell proliferation is decreased in *Wnt2b*-deficient mice

Adrenocortical development and homeostasis are both guided by Wnt/β-catenin signaling. As our global *Wnt2b* KO model provided significant insight into the necessity of WNT2B in adrenocortical development, we crossed *Wnt2b*-floxed and *Gli1-CreERT2* mice to generate a targeted conditional KO (cKO) model in which *Wnt2b* can be recombined specifically in *Gli1*-expressing cells of the adrenal capsule during adulthood to define the mechanisms by which WNT2B acts in homeostasis. Wild-type, heterozygous, and homozygous *Wnt2b*-floxed mice with or without *Gli1-CreERT2* were administered tamoxifen at 6 weeks of age. Genotyping from DNA samples of the intestines, which also contain *Gli1*/*Wnt2b*-expressing mesenchymal cells, confirms recombination of the floxed *Wnt2b* allele in *Gli1-CreERT2*-expressing mice 4 weeks post-treatments (wpt) **(Figure 2.8)**. Efficiency of the GLI1-CreERT2 driver was further assessed using an mTmG reporter. Upon activation in mTmG cells, Cre excises Tomato and allows for the expression of GFP, permanently turning cells from red to green. GFP-positive cells are seen throughout the adrenal capsule of tamoxifen-treated *Gli1-CreERT2*; *mTmG* mice, confirming driver activation **(Figure 2.9)**.

Figure 2.8. Validation of GLI1-CreERT2 activation by PCR
Intestinal DNA samples from *Gli1-CreERT2*; *Wnt2b*$^{fl/+}$ mice were genotyped using previously reported *Wnt2b* KO1 and Neo primers (Tsukiyama and Yamaguchi 2012). Wild-type and floxed *Wnt2b* products can be observed at 200 and 260 bp, respectively. Tamoxifen-treated (+TAM) sample also reveals a larger band at 320 bp (red arrowhead), validating *Wnt2b* gene recombination. L = ladder. Neg = negative water control. WT = *Wnt2b* +/+ control.

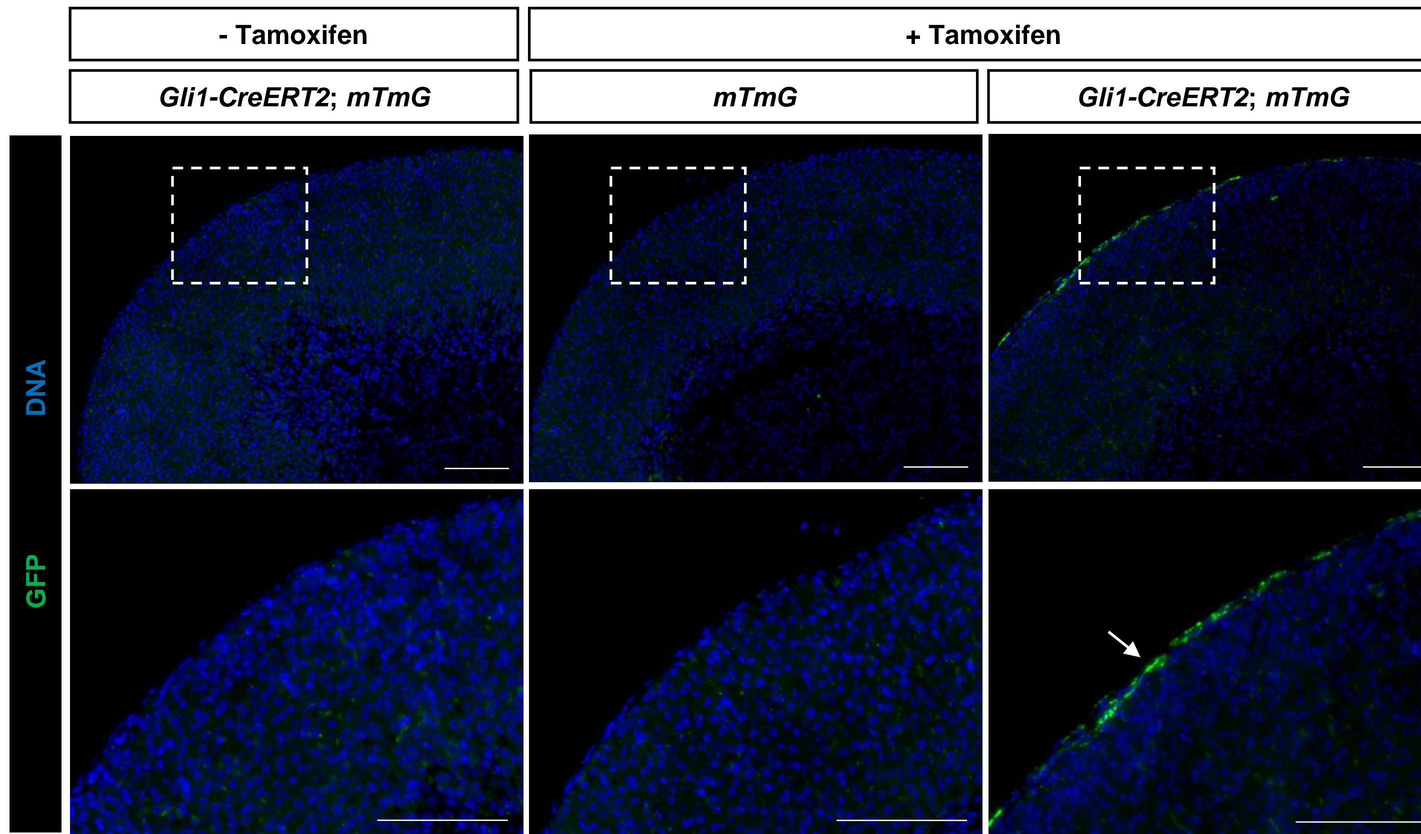


Figure 2.9. Capsular GLI1-CreERT2 activation using mTmG reporter
IF images of adrenal sections from mice harboring an *mTmG* reporter with or without tamoxifen-induced GLI1-CreERT2 activation. (Left and middle) No *mTmG* conversion is observed in untreated *Gli1-CreERT2*; *mTmG* or treated *mTmG* mice. (Right) GLI1-CreERT2 activity is observed in the capsule as marked by GFP expression (white arrow). Higher magnification shown in bottom images (denoted by white dashed boxes). Scale bar: 100 um.

GLI1-CreERT2 activation in *Wnt2b*$^{fl/fl}$ mice results in a significant (~70%) reduction in adrenal *Wnt2b* expression compared to Cre-negative *Wnt2b*$^{fl/fl}$ controls **(Figure 2.10)**. Adrenal weights were measured as an initial assessment of the effect of *Wnt2b* loss. Adrenal-to-body weight ratios of *Wnt2b* cKO mice are significantly lower than their control counterparts **(Figure 2.11)**. Reduction in adrenal weights could be due to either

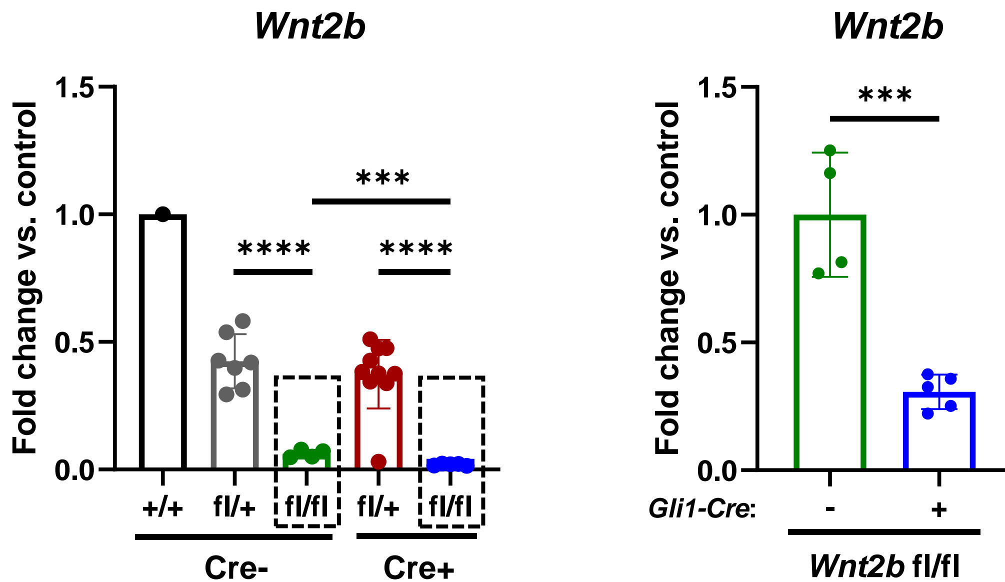


Figure 2.10. GLI1-CreERT2 activation significantly reduces *Wnt2b* expression
qPCR on adrenal cDNA from tamoxifen-treated male mice. (Left) Comparison of mice with or without GLI1-CreERT2 activation based on *Wnt2b* genotype. Significant decreases in *Wnt2b* expression are observed in heterozygous (fl/+)

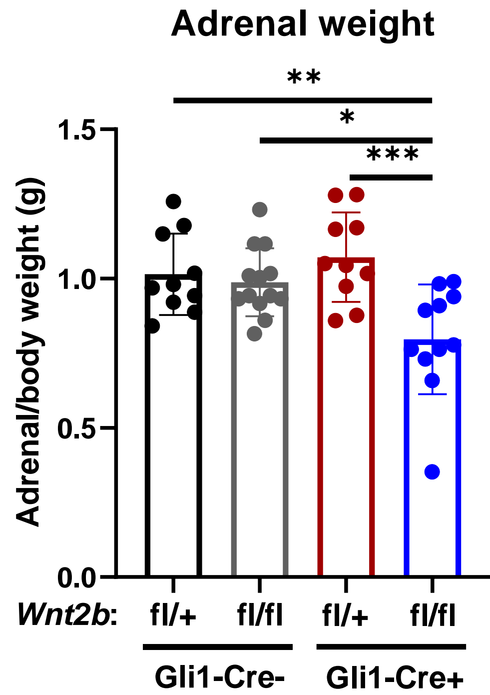


Figure 2.11. *Wnt2b* cKO mice have reduced adrenal-to-body weight ratios
Adrenal weights from 10-week-old tamoxifen-treated *Wnt2b* fl/+ and fl/fl male mice with or without *Gli1-CreERT2* were measured and normalized to body weight. Statistics performed using one-way ANOVA; *p<0.05; **p<0.01; ***p<0.001.

reduced proliferation or increased cell death. To determine the cellular mechanism involved, I then assessed KI67 (proliferation) and cleaved caspase 3 (death) levels by IHC. While there is no increase in cell death at the time point analyzed (data not shown), KI67+ proliferative cells are decreased in *Wnt2b* cKO mice by quantification per high power field (IHC) and in the zG alone (DAB2/KI67 co-IF) **(Figure 2.12)**. Together, these data suggest that WNT2B ligands secreted by *Gli1*-expressing cells of the capsule play an important role in adrenocortical cell proliferation.

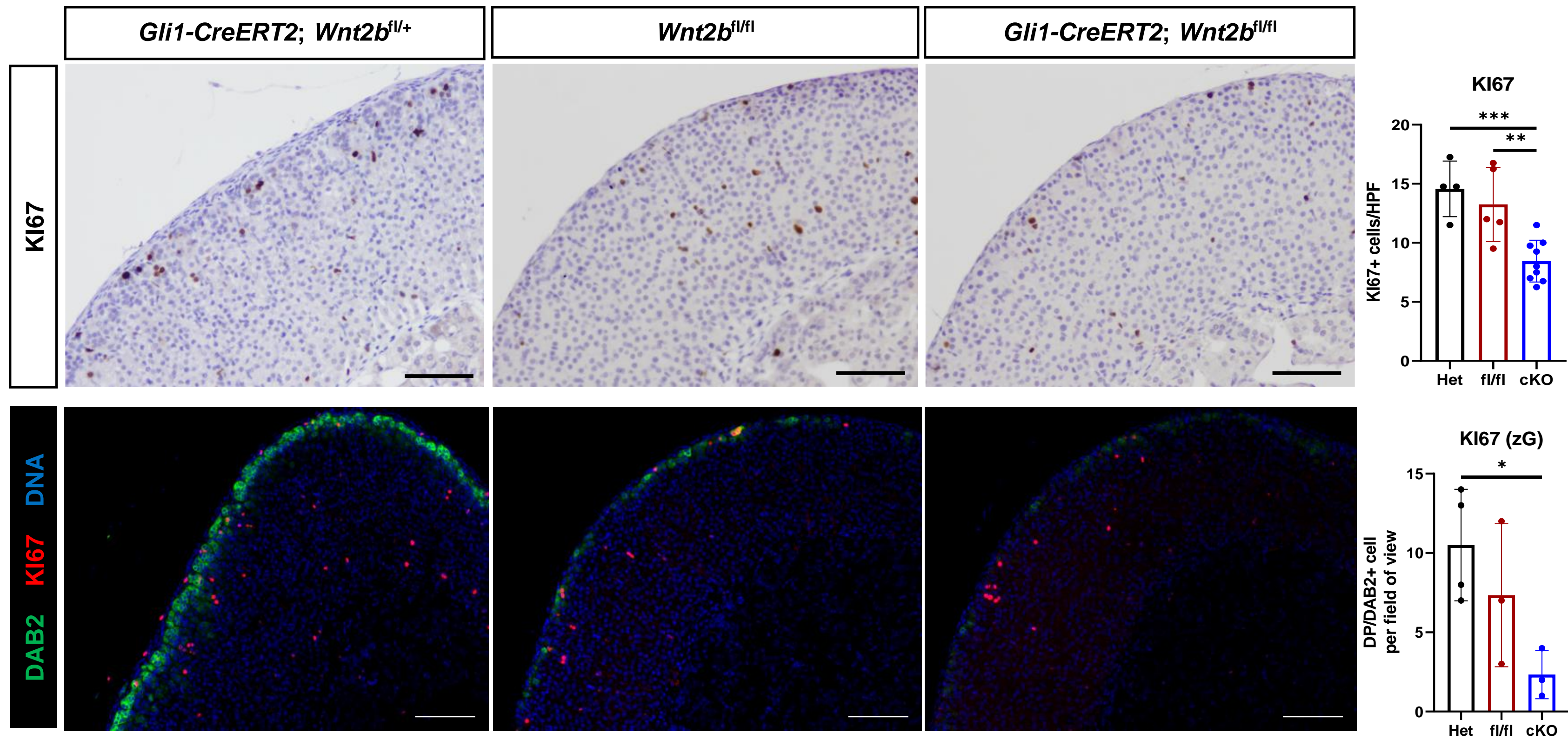


Figure 2.12. *Wnt2b* loss results in decreased adrenocortical cell proliferation
(Top) Representative of adrenocortical KI67 IHC in *Wnt2b* control and cKO mice. Quantification (right) shows significant reduction in proliferation in *Wnt2b* cKO mice compared to controls. (Bottom) DAB2/KI67 co-IF images show decreased cell proliferation in the zG of *Wnt2b* cKO adrenals. Student's t test; *p<0.05. Scale bar: 100 um.

2.8 WNT2B regulates adrenocortical Wnt-high activity

WNT2B has been defined as a canonical WNT ligand due to its capacity to activate β-catenin and downstream transcriptional programs in Wnt-responsive cells (H. Liu et al., 2003; Iglesias et al., 2007; Goss et al., 2009; DiRenzo et al., 2016; Yuan et al., 2017). β-catenin is essential for proper adrenocortical zonation and maintenance as mice lacking *Ctnnb1* have late-stage adrenal failure, suspected to be due to diminishment of the zG-restricted progenitor cell pool (Kim, A.C. et al., 2008). Given these data along with the decreased adrenocortical proliferation in *Wnt2b* cKO mice, I hypothesized that WNT2B activates canonical Wnt/β-catenin signaling in the adrenal cortex. To test this, I first analyzed β-catenin activity in the adrenal cortex of *Wnt2b*-deficient mice. Wild-type mice have a zG-to-zF Wnt/β-catenin activity gradient as observed by IHC for active β-catenin (Basham et al., 2019). However, this gradient is largely disrupted in *Wnt2b* cKO mice **(Figure 2.13)**. While small patches of the cortex retain normal β-catenin activity, the majority of the zG has lower levels of β-catenin activity more closely resembling Wnt-moderate cells of the upper zF. This supports our hypothesis that WNT2B directly activates β-catenin in the subcapsular zG.

To determine the extent to which the Wnt/β-catenin gradient is affected in *Wnt2b*-deficient mice, I next performed qPCR and single molecule *in situ* hybridization (smISH, or RNAScope) on β-catenin target genes (qPCR data not shown). Quantification of *Axin2* and *Wnt4* transcripts shows that both are significantly downregulated in the context of *Wnt2b* loss **(Figure 2.14)**. Moreover, *Wnt2b*-deficient adrenals have significantly lower expression of Wnt/β-catenin target LEF1 compared to controls as assessed by

immunofluorescence **(Figure 2.14)**. As RNAScope allows for both the quantification and zonal visualization of transcripts in the adrenal cortex, I then assessed the abundance of *Wnt4* transcripts in subsequent centripetal regions of the cortex to determine whether zG cells of *Wnt2b*-deficient mice have β-catenin activity more similar to that of the upper zF in control mice. *Wnt4* ISH was quantified in R1 (zG), R2 (upper zF), and R3 (inner zF) regions for comparison. Indeed, while significant differences in *Wnt4* expression exist between centripetal regions within genotypes, *Wnt4* quantification in the zG (R1) of *Wnt2b* cKO mice is significantly less than corresponding R1 but not R2 (upper zF) measurements in heterozygous and *Wnt2b*$^{fl/fl}$ mice, indicating that zG *Wnt4* expression in *Wnt2b* cKO mice more closely resembles that of the zF in control mice (Basham et al., 2019) **(Figure 2.15)**. These data support a direct role for WNT2B in regulating Wnt-high activity in the zG, in part through activating the β-catenin-dependent transcription of *Wnt4* in a Wnt/β-catenin signaling cascade.

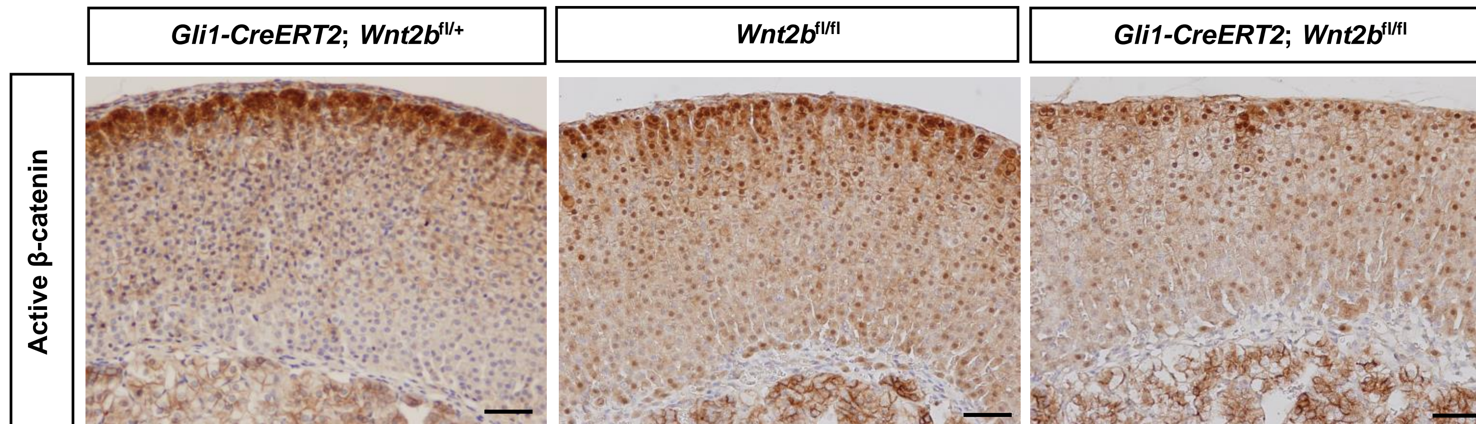


Figure 2.13. β-catenin activity gradient is disrupted in *Wnt2b*-deficient adrenals
Representative IHC images of active (non-phosphorylated) β-catenin shows disrupted Wnt-high activity in the zG of *Wnt2b* cKO adrenals. Control adrenals have expected dense nuclear and cytoplasmic β-catenin staining in the zG, which then decreases centripetally into the zF. *Wnt2b* cKO adrenals lack this high zG β-catenin staining. Scale bar: 100 um.

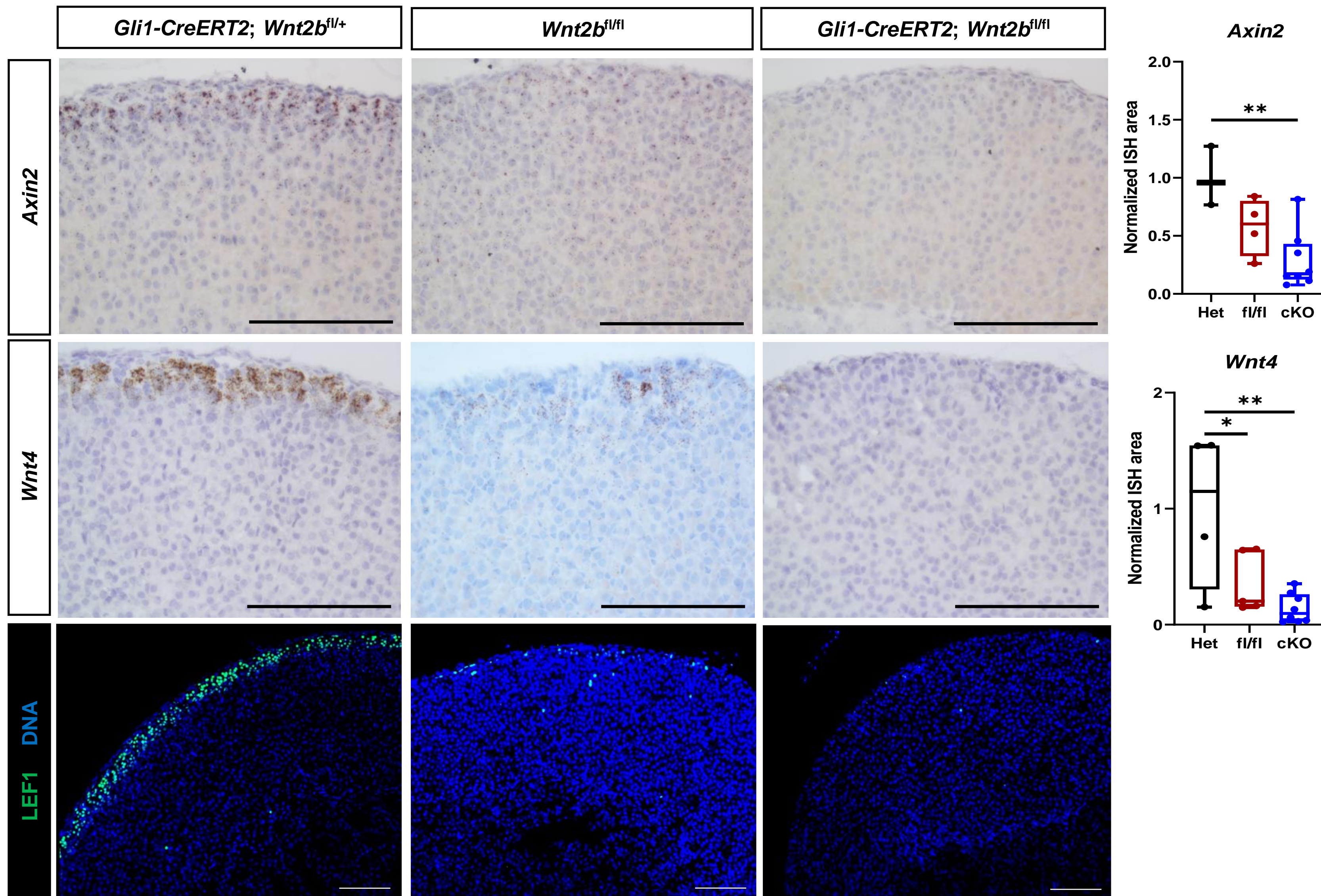


Figure 2.14. *Wnt2b* loss results in downregulation of Wnt/β-catenin target genes
Representative RNAScope ISH images of *Axin2* (top) and *Wnt4* (middle) expression in *Wnt2b* heterozygous, fl/fl, and cKO adrenals. Quantification shows significant decreases in the adrenal cortex of *Wnt2b* cKO mice. Statistics performed using ordinary one-way ANOVA; *p<0.05; **p<0.01. (Bottom) IF staining reveals a concurrent downregulation of β-catenin target gene LEF1 in *Wnt2b*$^{fl/fl}$ and cKO mice. Scale bar: 100 um.

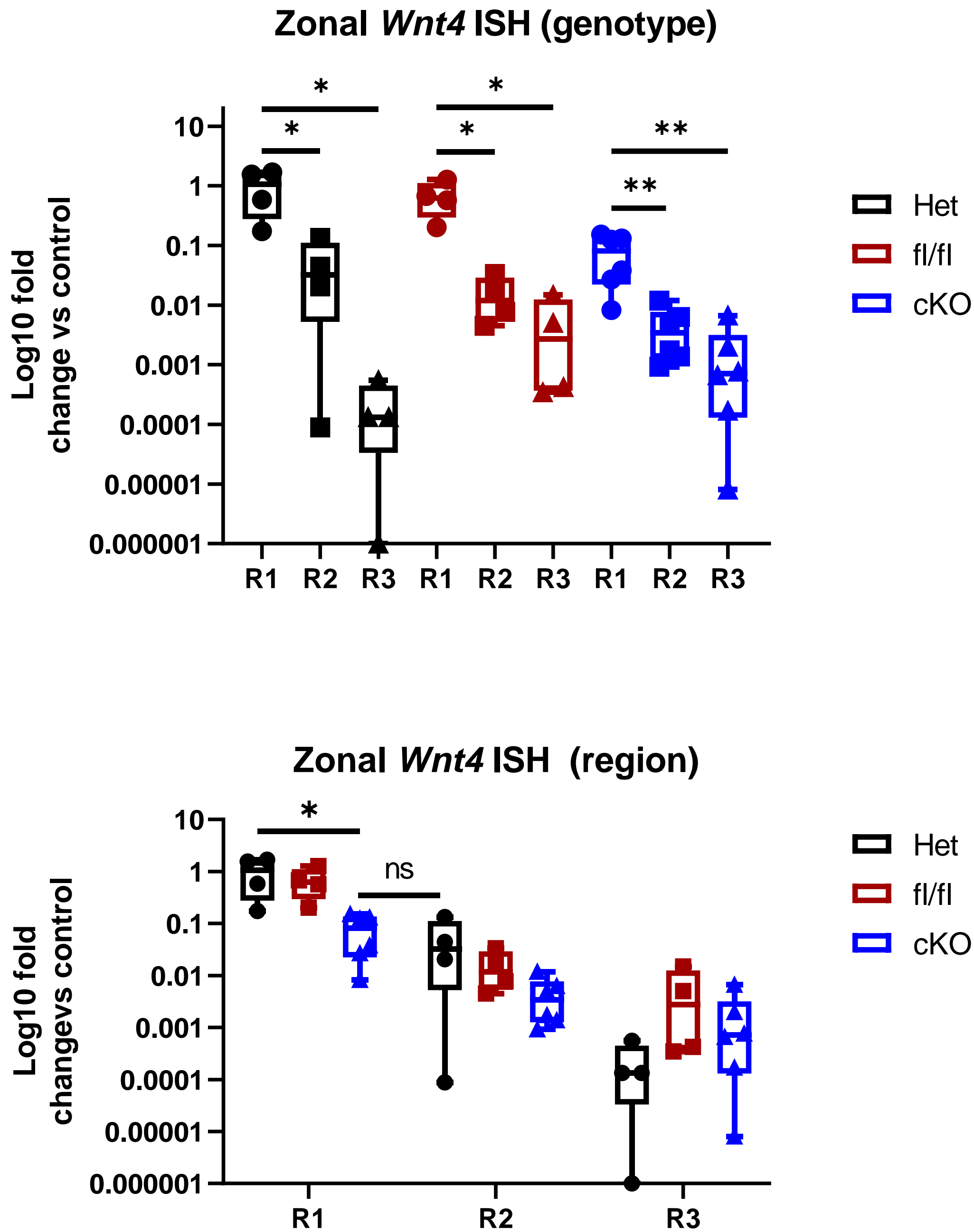


Figure 2.15. Zonal *Wnt4* quantification in *Wnt2b* mice
Wnt4 RNAScope was quantified in three centripetal regions from the capsule inward—R1 (zG), R2 (upper zF), and R3 (inner zF)—in treated *Wnt2b* heterozygous (Het), fl/fl, and cKO mice. Data are grouped to show differences between genotypes (top) and regions (bottom). Significant differences in *Wnt4* abundance are shown in R1 vs R2 and R1 vs R3 comparisons within genotypes. The R1 region of *Wnt2b* cKO adrenals shows significantly less *Wnt4* expression and more closely resembles R2 of *Wnt2b* heterozygous mice (R1 blue vs. R2 black; labeled “ns”). Intragenotype and intraregion statistics performed using one-way ANOVA; ns = not significant; *p<0.05; **p<0.01.

2.9 *Wnt2b* deficiency in mice phenocopies renin-mediated aldosterone compensation observed in *WNT2B*-null patients

Wnt/β-catenin signaling is essential for both the zonation and function of the adrenal cortex. While mice lacking β-catenin have disrupted adrenocortical zonation, *Wnt4* KO mice have normal zonation but decreased levels of aldosterone (Kim, A.C. et al., 2008; Heikkilä et al., 2002). Through activation of *Wnt4* expression, adrenocortical Wnt/β-catenin signaling therefore plays an essential role in the renin-angiotensin-aldosterone system (RAAS). Given these data, the downregulation of *Wnt4* in *Wnt2b*-deficient mice begged the question if RAAS-associated aldosterone production was also disrupted. Based on the necessity of WNT2B and Wnt/β-catenin signaling activity for zG development shown by our data and previous studies, I assessed overall zG morphology in *Wnt2b* heterozygous, *Wnt2b*$^{fl/fl}$, and cKO adrenals. IF staining of DAB2, a robust zG marker, shows markedly disrupted zG morphology in *Wnt2b*-deficient mice **(Figure 2.16)**. Moreover, *Wnt2b* loss seems to result in disorganization of zG rosettes, a stem/progenitor cell niche structure regulated by Wnt/β-catenin in the adrenal cortex that is important for proper aldosterone synthesis (Leng et al., 2020).

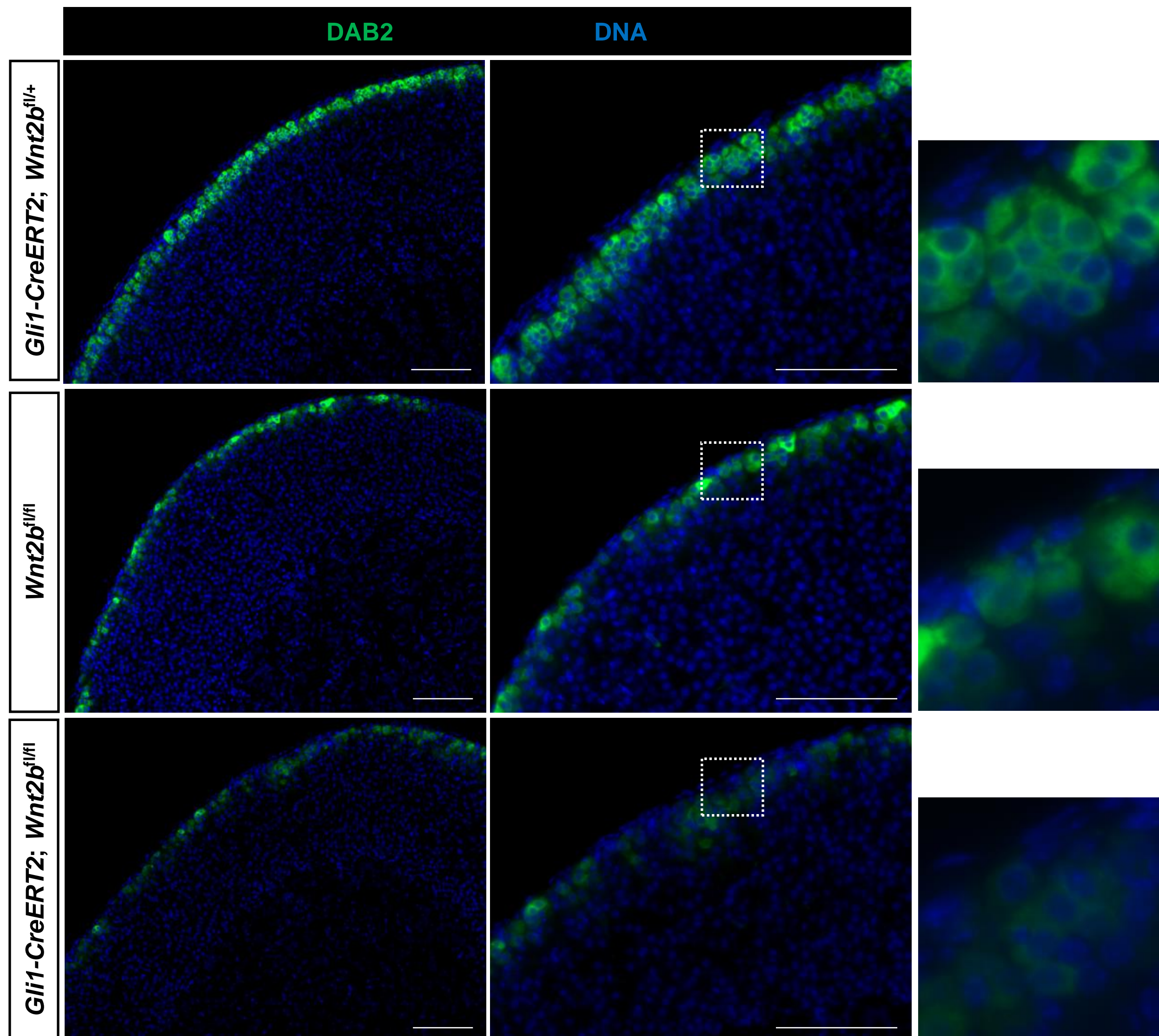


Figure 2.16. *Wnt2b*-deficient adrenals exhibit disorganized zG morphology
(Left and middle) IF images of adrenocortical DAB2 marking the entirety of the zG in *Wnt2b* heterozygous (top) compared to *Wnt2b*$^{fl/fl}$ (middle) and cKO (bottom) mice. *Wnt2b*-deficient mice exhibit patchy DAB2 expression, indicating zG loss and/or disorganization. Higher magnification is shown in middle images. (Right) Magnified portion of zG rosette (white dashed box in middle panels). Note organized cell cluster in top image, whereas this typical zG rosette morphology is diminished in *Wnt2b* fl/fl and cKO adrenals. Scale bar: 100 um.

Given these data, I hypothesized that disrupted zG structure in *Wnt2b*-deficient mice would have a subsequent negative effect on zG marker gene expression and downstream function. To assess the expression of genes characteristic of the zG, I first performed RNAScope on *Cyp11b2* and *Shh*. While a downward trend is observed in the expression of *Cyp11b2*, the terminal enzyme in aldosterone synthesis, *Wnt2b* cKO adrenals have significantly lower abundance of *Shh*, a progenitor cell marker **(Figure 2.17)**. Parallel IHC experiments show CYP11B2 expression present in few cell clusters throughout the subcapsular region of *Wnt2b*-deficient mice. While small portions of the cortex of *Wnt2b* fl/fl mice retain normal CYP11B2 expression patterns, downregulation of CYP11B2 is significantly more pronounced in *Wnt2b* cKO mice. Interestingly, where CYP11B2 expression is absent, cells expressing AKR1B7, a zF marker believed to be involved in lipid detoxification, are present. These results suggest that WNT2B activity maintains the expression of both progenitor and aldosterone-producing cell markers in the zG, likely through the mediation of Wnt-high activity that is necessary for proper zG identity, structure, cortex renewal, and RAAS engagement.

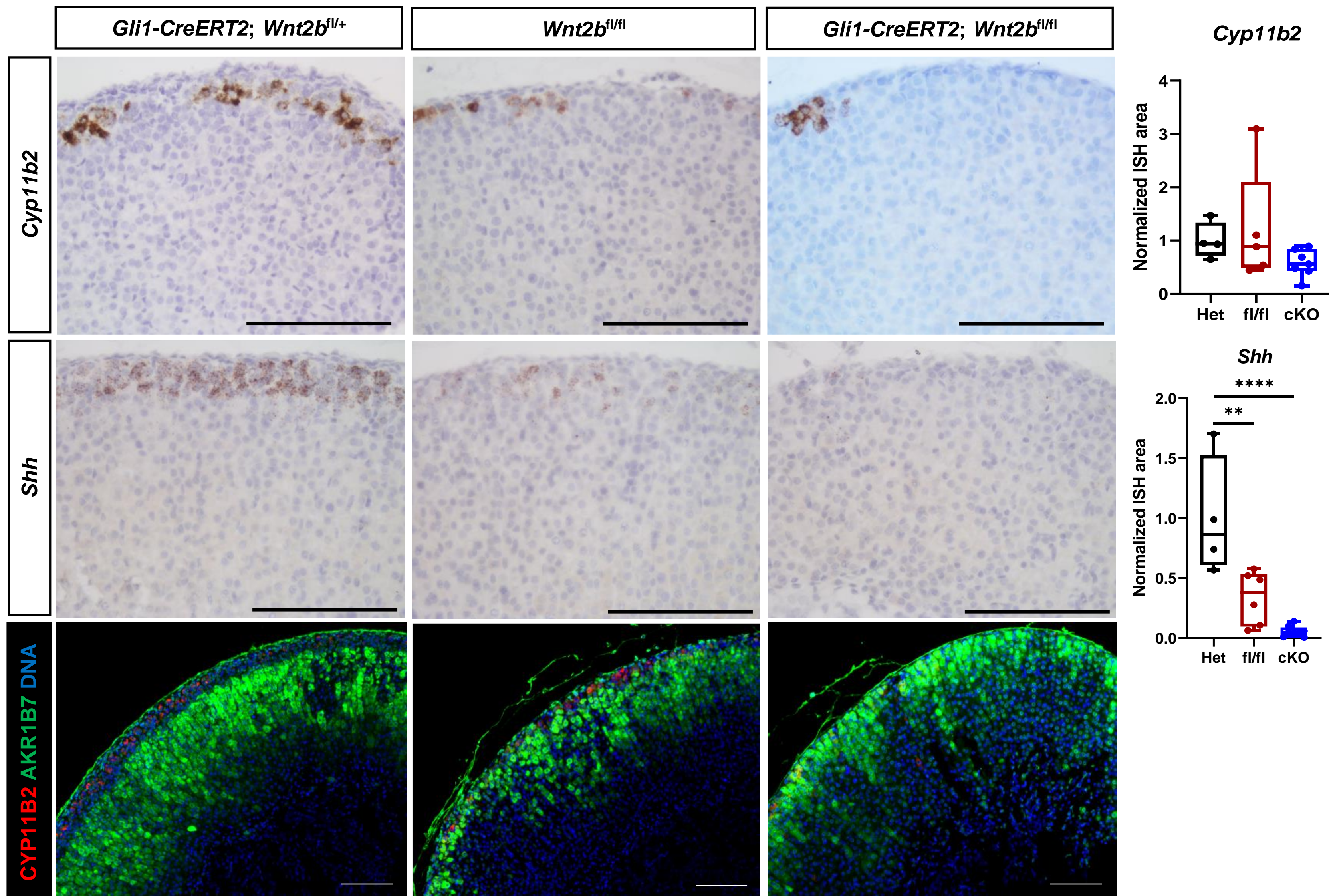


Figure 2.17. ***Wnt2b*** **loss results in zG marker downregulation and disrupted zonation**
Representative RNAScope ISH images of *Cyp11b2* (top) and *Shh* (middle) expression in *Wnt2b* heterozygous (*Gli1-CreERT2*; *Wnt2b* fl/+), fl/fl, and cKO adrenals and quantification. One-way ANOVA; **p<0.01; ****p<0.0001. (Bottom) Co-IF staining for CYP11B2 (zG) and AKR1B7 (zF) shows profound disruption of normal zG organization and morphology in *Wnt2b*-deficient mice. Scale bar: 100 um.

To further define the effect of *Wnt2b* loss on the engagement of zG cells in the RAAS, I next assessed aldosterone levels in tamoxifen-treated *Wnt2b*$^{fl/+}$, *Wnt2b*$^{fl/fl}$, heterozygous, and cKO male mice. However, no significant differences in aldosterone

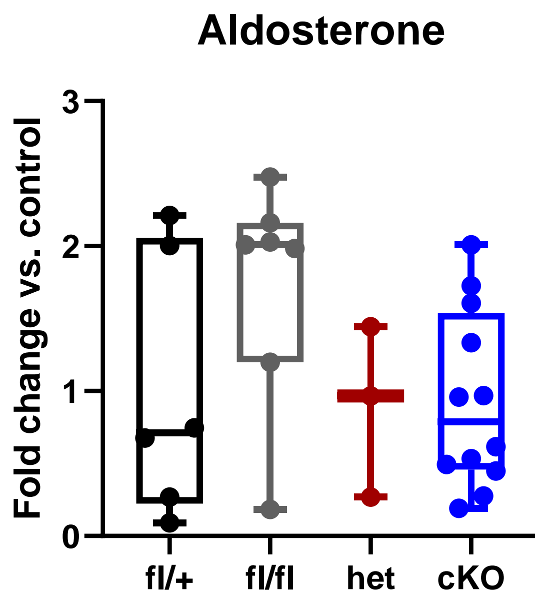


Figure 2.18. Aldosterone levels in *Wnt2b*-floxed mice
Plasma aldosterone levels were measured in 10-week-old tamoxifen-treated *Wnt2b* mice of various genotypes by LC/MS-MS. No significant differences were found between groups. Statistics performed using one-way ANOVA.

levels are present **(Figure 2.18)**. While at first surprising, significantly reduced aldosterone is not observed in *WNT2B*-null patients due to elevated renin levels that compensate for hypoaldosteronism caused by WNT2B loss. Furthermore, only basal aldosterone levels in *Wnt2b* mice were analyzed here, whereas *WNT2B*-null patients are subject to chronic hypovolemic stress that affects basal adrenal function. Therefore, I hypothesized that the effect of WNT2B loss on adrenocortical RAAS engagement would be augmented in the presence of stress. To test this, I treated 4-6-month-old WT and *Wnt2b*$^{fl/fl}$ mice with a sodium-deficient (SD) diet, which causes subsequent physiological increases in adrenocortical *Cyp11b2* expression and plasma aldosterone levels (Holmer

et al., 1993). Indeed, *Wnt2b* fl/fl mice have significantly reduced *Cyp11b2* expression but normal plasma aldosterone at baseline (normal salt) **(Figure 2.19)**. Administration of SD diet to WT and *Wnt2b*$^{fl/fl}$ mice leads to significant yet proportional increases in *Cyp11b2* (7-fold each) and plasma aldosterone (16-fold and 14-fold, respectively). Considering the proportional increases in aldosterone, I then assessed renin levels to test the hypothesis

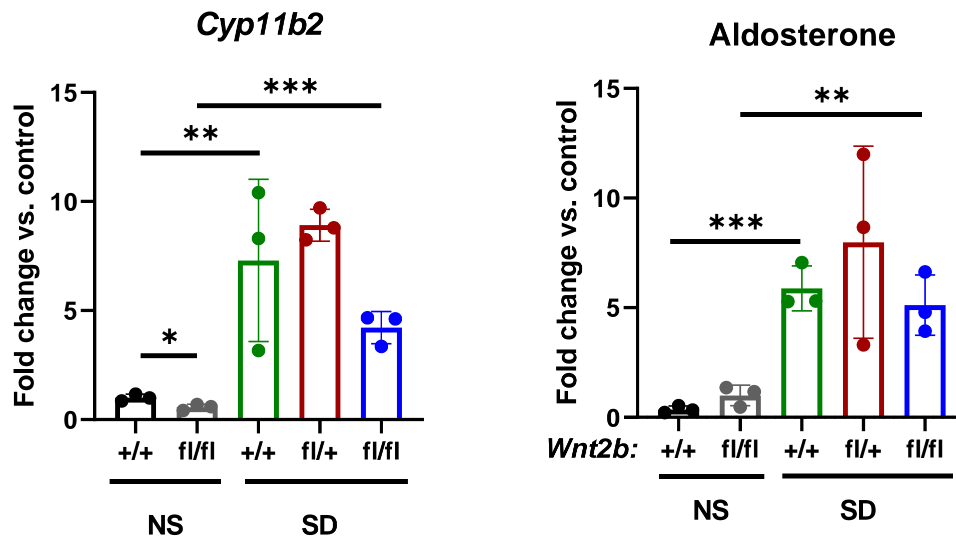


Figure 2.19. Proportional increases in RAAS engagement in *Wnt2b*$^{fl/fl}$ mice under physiological stress
Adult *Wnt2b* +/+, fl/+, and fl/fl mice were treated with normal salt (NS) or sodium deficient (SD) diet for one week. SD mice exhibit a significant increase in adrenocortical *Cyp11b2* expression (qPCR, left) and plasma aldosterone (LC/MS-MS, right). *Wnt2b* +/+ and fl/fl mice both exhibit a 7-fold increase in *Cyp11b2* on SD diet. Aldosterone production increases 16-fold and 14-fold in Wnt2b +/+ vs fl/fl mice, respectively. Statistics performed using Student's t test and one-way ANOVA; *p<0.05; **p<0.01; ***p<0.001.

that *Wnt2b*-deficient mice exhibit elevated renin levels to compensate for dysfunctional aldosterone production under stress. Indeed, while renin levels in *Wnt2b*$^{fl/fl}$ mice are comparable to WT at baseline, they have significantly increased kidney *Ren1* expression and plasma renin levels on SD diet **(Figure 2.20)**. **These data strongly suggest that capsular *Wnt2b* loss results in renin-mediated aldosterone compensation in the presence of physiological stress.** Thus, our model can be used to sufficiently recapitulate the phenotype of renin-mediated aldosterone compensation in *WNT2B*-null human patients and provides an avenue to further study the role of Wnt-high signaling in adrenocortical steroidogenesis and progenitor cell biology.

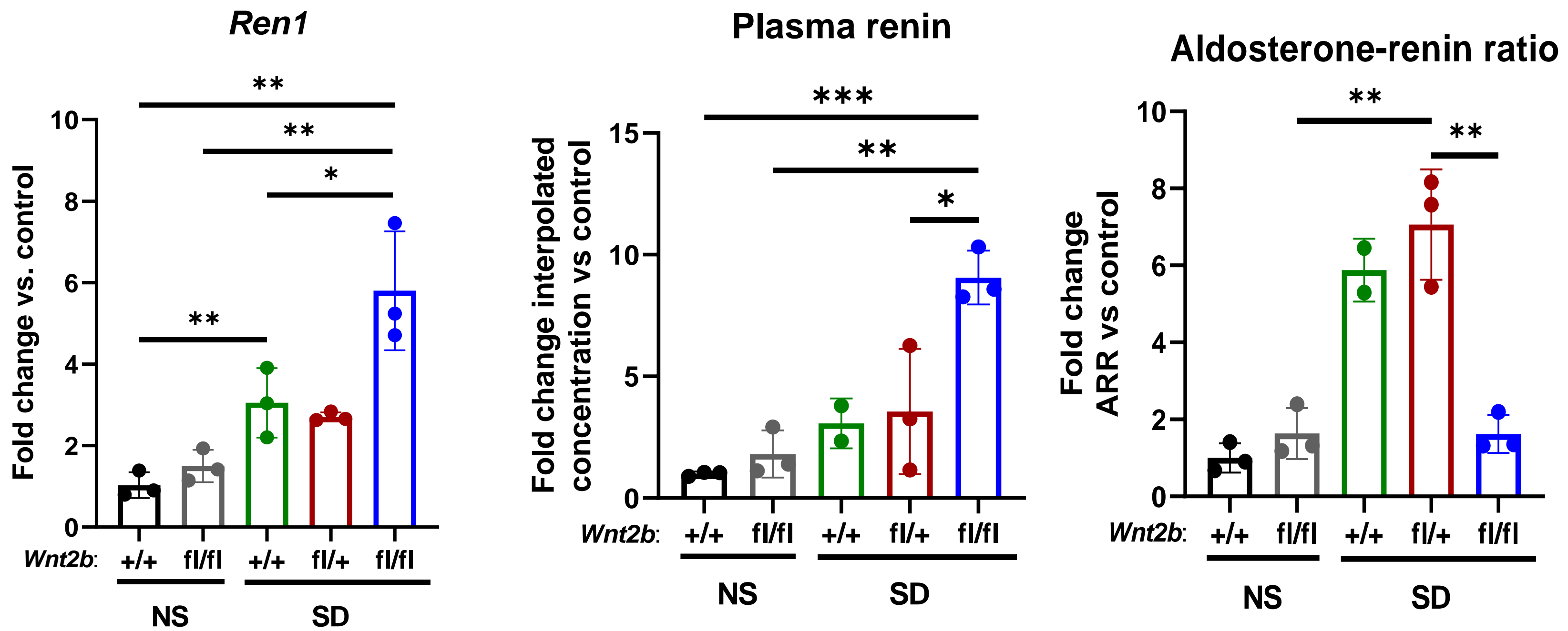


Figure 2.20. Sodium deficiency unmasks renin-mediated aldosterone compensation in *Wnt2b*$^{fl/fl}$ mice
Renin levels in Wnt2b +/+, fl/+, and fl/fl mice treated with normal salt (NS) or sodium deficient (SD) diet were assessed by qPCR on kidney *Ren1* (left) and mouse plasma renin ELISA (middle). SD diet results in significant increases in renin levels in both +/+ and fl/fl mice compared to NS. However, Wnt2b fl/fl mice on SD diet have significantly elevated renin levels compared to control mice, indicating compensation. (Right) Aldosterone-renin ratios (ARR) are significantly lower in *Wnt2b* fl/fl mice compared to controls on SD diet. Statistics performed using Student's t test or one-way ANOVA; *p<0.05; **p<0.01; ***p<0.001.

2.10 Single-cell RNA sequencing of adrenocortical Wnt-GFP cells reveals cell-specific transcriptional programs

The adrenal zG is a heterogenous population of undifferentiated progenitor and differentiated/aldosterone-producing cells. Previous work in our lab utilizing FACS enrichment of GFP-labeled Wnt-responsive adrenocortical cells (*Tcf/Lef:H2B-GFP*, referred to here as Wnt-GFP) established a paradigm for defining Wnt/β-catenin target genes in different zG cell populations (Walczak et al., 2014). RNA sequencing on primary Wnt-GFP cells and cultured adrenocortical cells treated with a β-catenin-activating drug revealed *bona fide* adrenocortical β-catenin target genes. However, while the presence of nearly mutually exclusive undifferentiated *Shh*-expressing progenitors and differentiated *Cyp11b2*-expressing cells was confirmed, the technology employed precluded our ability to determine cell-specific Wnt/β-catenin target genes. Since both zG cell populations are Wnt-responsive, we found it of great importance to identify β-catenin target genes unique to progenitor and steroid-producing zG cells to gain greater insight into the cell-specific transcriptional programs engaged by Wnt/β-catenin signaling and their role in progenitor cell maintenance, fate determination, and steroidogenesis.

To distinguish between cell-specific Wnt/β-catenin transcriptional programs in the zG, we utilized single cell RNA sequencing (scRNAseq) on adult Wnt-GFP mouse adrenals (schematized in **Figure 2.21**). 1,551 adrenocortical Wnt-GFP cells were sorted by FACS and analyzed by scRNAseq **(Figure 2.22)**. *Sf1* (*Nr5a1*) expression validated the

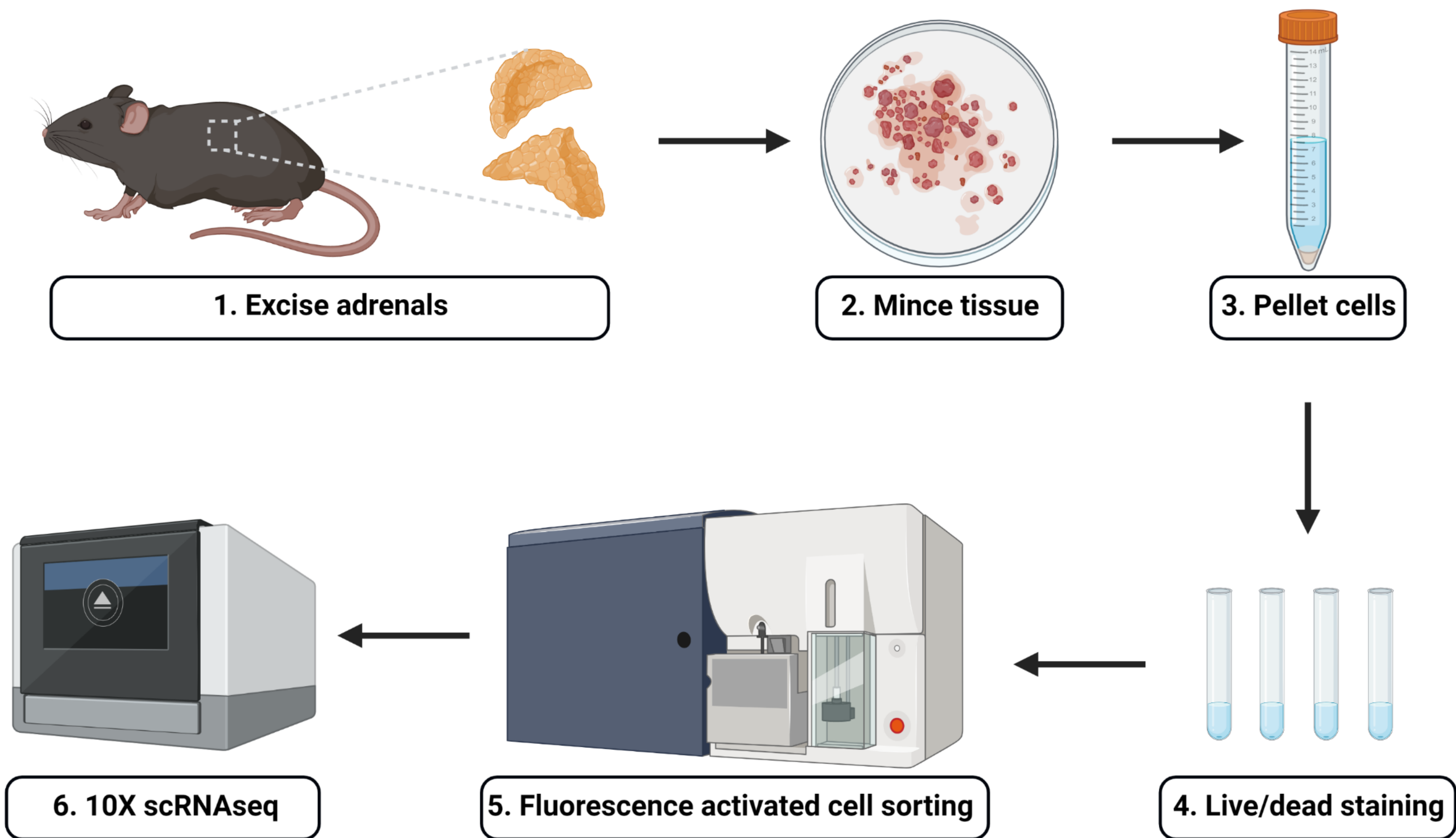


Figure 2.21. Schematic of single cell RNA sequencing (scRNAseq) workflow

enrichment of adrenocortical cells **(Figure 2.23)**. Moreover, K-means clustering reveals two major Wnt-responsive cell populations of the zG (Cluster 1) and upper zF (Cluster 2). The zG can be observed by concentrated cell clusters expressing high levels of *Cyp11b2* and *Wnt4*, the latter of which decreases gradually in cells of the zF marked by high *Cyp11b1* expression **(Figure 2.23)**. To our surprise, we observe *Shh* expression throughout the zG, whereas expression of secreted SHH ligands have previously been shown to be exclusive to undifferentiated zG progenitor cells **(Figure 2.24)**. The post-transcriptional and translational regulation of *Shh* in zG cells thus requires further study (discussed in **Chapter 4**).

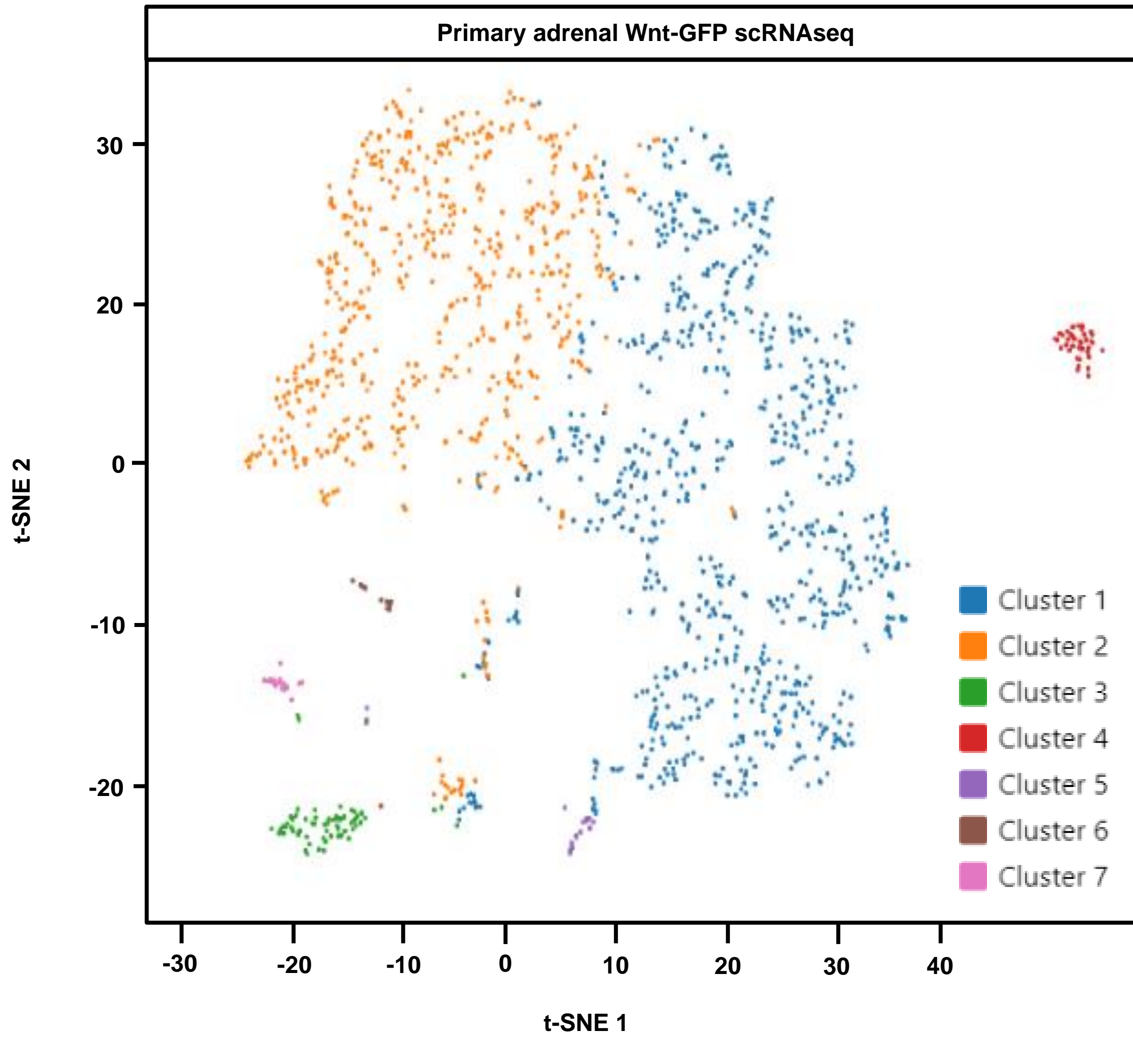


Figure 2.22. scRNAseq on primary adrenocortical Wnt-GFP cells
Plot depicting 1,551 primary adrenocortical Wnt-GFP cells analyzed by scRNAseq. Single cells are depicted as dots and are plotted by neighbor embedding such that cells closest to one another express more similar transcriptomes. K means clustering reveals 7 unique cell clusters. Cluster 1 (blue, right) and Cluster 2 (orange, left) represent zG and upper zF, respectively, as analyzed in following figures.

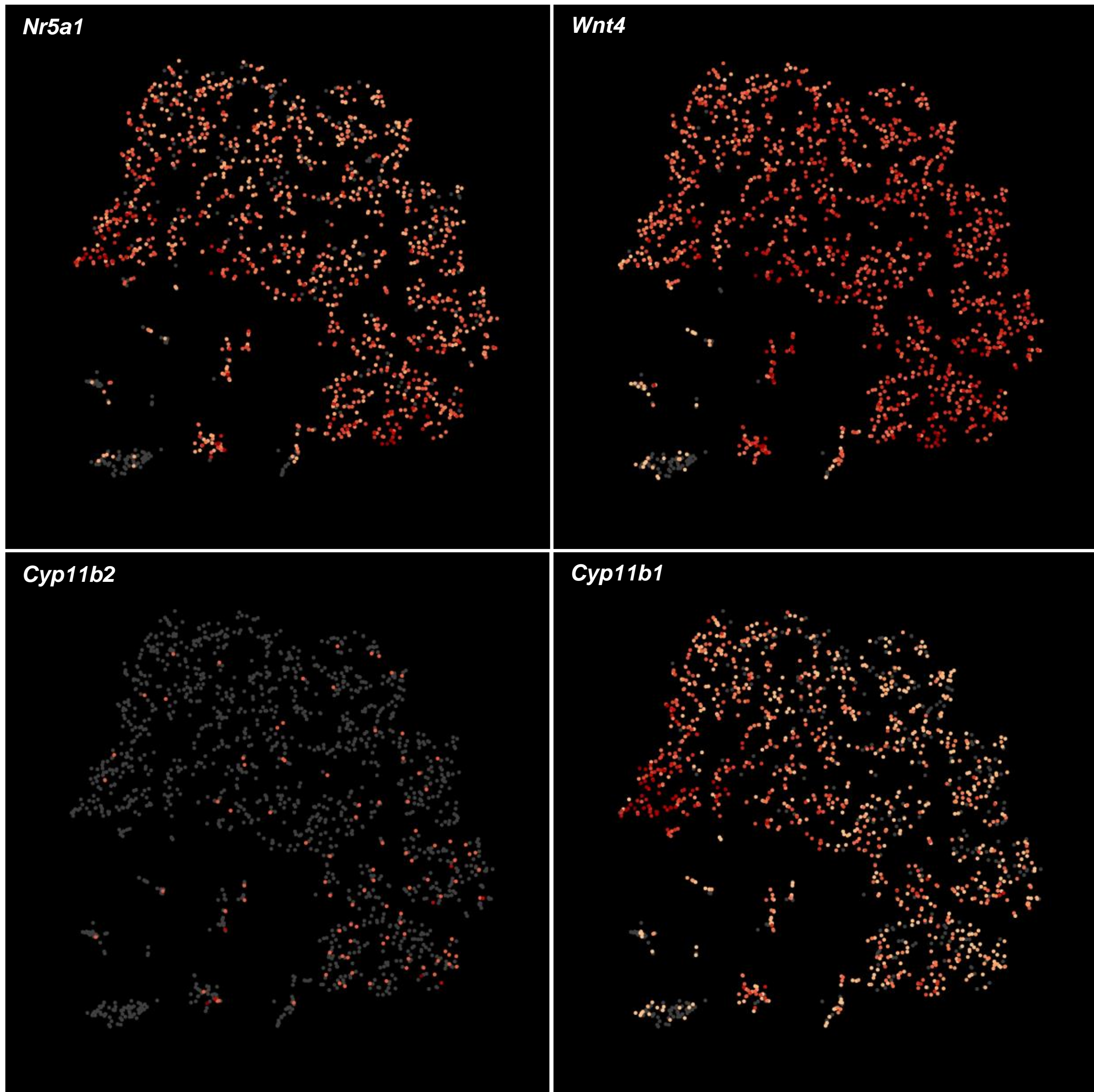


Figure 2.23. Relevant Wnt-GFP scRNAseq t-SNE plots
t-SNE plots showing genes of interest expressed in 1,551 sorted primary adrenocortical Wnt-GFP cells. Isolation and scRNAseq on Wnt-responsive adrenocortical cells is verified by robust *Sf1* (*Nr5a1*) (top left) and *Wnt4* (top right) expression. Note the right to left gradient of *Wnt4* expression representative of the adrenocortical Wnt/β-catenin signaling gradient. High *Wnt4* expression corresponds to *Cyp11b2*-high cells of the zG (bottom left) while lower *Wnt4* is observed in *Cyp11b1*-high cells of the upper zF (bottom right).

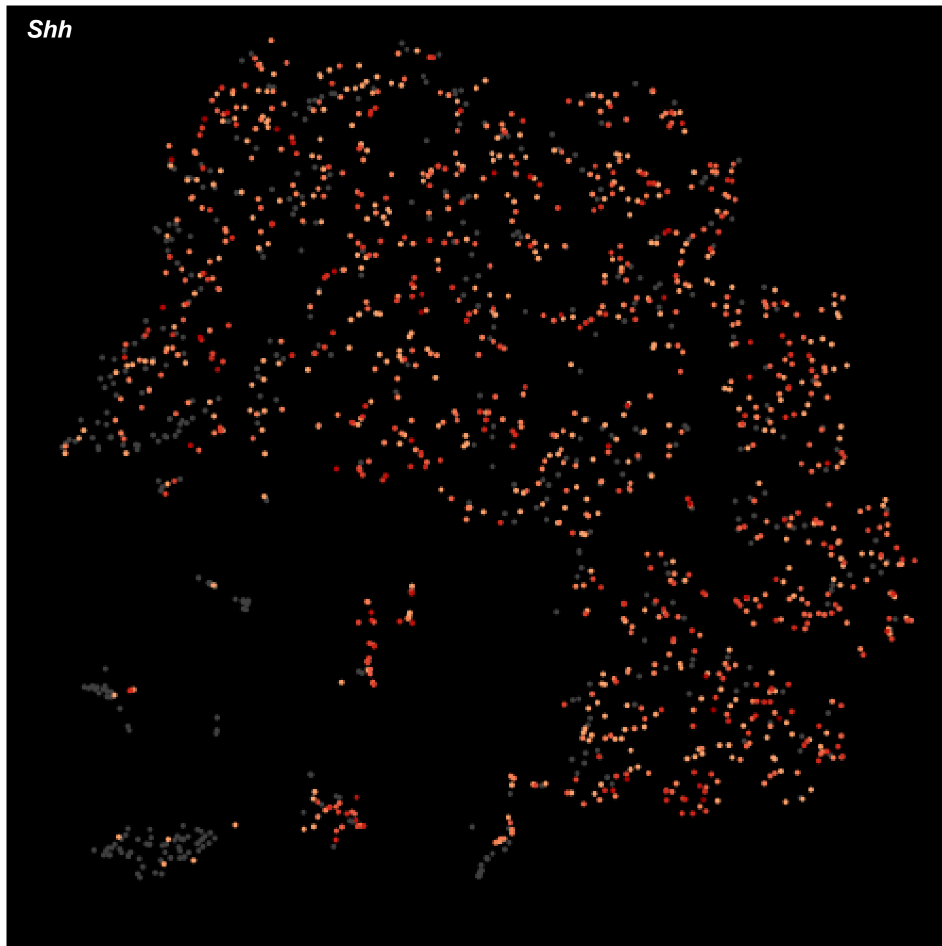


Figure 2.24. Wnt-GFP cell *Shh* expression
scRNAseq reveals *Shh* expression throughout the adrenocortical Wnt-GFP cell population, although the mechanisms regulating its transcriptional and translational regulation in specific zG cells are unknown.

To determine unique transcripts expressed by aldosterone-producing cells of the zG, I first analyzed *Cyp11b2*-high cells of Cluster 1 in comparison to all other cells. Gene set enrichment analysis (GSEA) on the 100 most significantly upregulated genes revealed transcription modules involved in stem cell biology and response to estrogen and progesterone, among others **(Figure 2.25)**. Transcripts most significantly upregulated in *Cyp11b2*-high cells include *Gulo*, *Pde10a*, *Vsnl1*, *Ism1*, *1500015O10Rik* (*Ecrg4*), and *Agtr1a/b* **(Table 2.2)**. Many transcripts follow the same zG-to-zF expression gradient as *Wnt4* and show a downward trend in expression in *Wnt2b* cKO mice compared to controls **(Figure 2.26)**. Additionally, GSEA analysis was performed on *Ki67*-positive

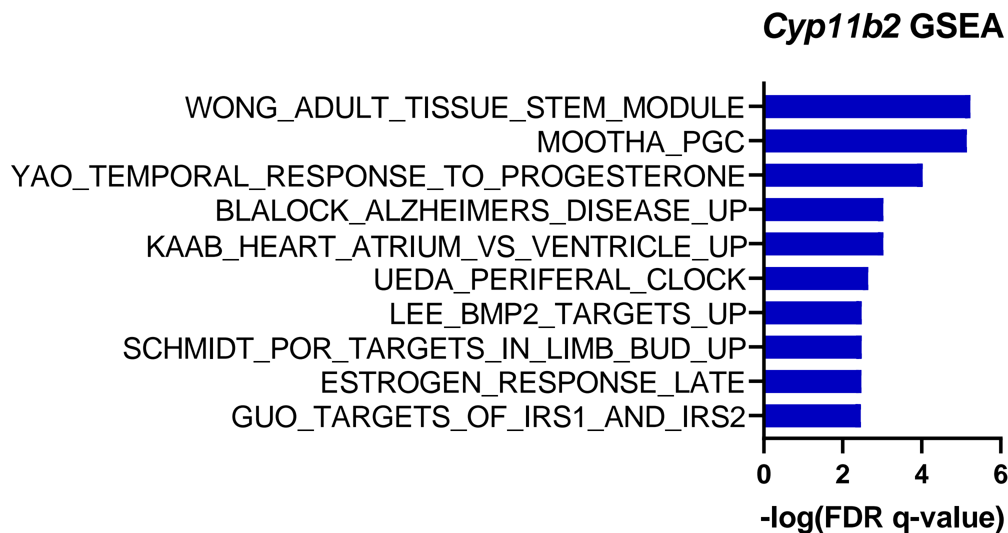


Figure 2.25. GSEA of *Cyp11b2*-high Wnt-GFP scRNAseq genes
Gene set enrichment analysis (GSEA) on the top 100 genes significantly upregulated in *Cyp11b2*-high Wnt-GFP cells analyzed by scRNAseq.

Table 2.2.Significantly upregulated genes in *Cyp11b2*-high Wnt-GFP cells analyzed by scRNAseq

Gene	Log2 Fold Change	P Value	Function/description
Gulo	2.08151693	2.26E-06	L-gulonolactone oxidase; Vitamin C production
Pde10a	1.813415062	8.49E-06	cAMP-associated phosphodiesterase
Fdx1	1.744759921	1.47E-05	Ferredoxin 1- mitochondrial electron transfer in steroid metabolism
Col18a1	1.804676725	1.62E-05	Collagen 18a1 (ECM)
Nsf	1.585542733	1.16E-04	Vesicle-mediated transport
P2ry14	1.565758407	2.93E-04	GPCR for UDP-sugars; stem cell compartment, immune function
Vsnl1	1.507613979	3.04E-04	Neuronal calcium ion binding
Elovl6	1.477012261	5.47E-04	Fatty acid elongation
Agtr1b	1.389639226	1.46E-03	Angiotensin II receptor
1500015O10Rik	1.323866999	2.06E-03	Esophageal cancer-related gene protein 4 (ECRG4)- cell proliferation and senescence (predicted)
Ism1	1.341432773	2.15E-03	Wnt target gene
Steap1	1.271627321	4.88E-03	Prostate cell-cell junction antigen
Tspan6	1.188444783	1.21E-02	Negative regulator of immune activity
Agtr1a	1.144205821	1.21E-02	Angiotensin II receptor
Zfand5	1.145474903	1.21E-02	Ubiquitin-mediated protein degradation
Rgs4	1.149021618	1.36E-02	GAP (G-protein activator)

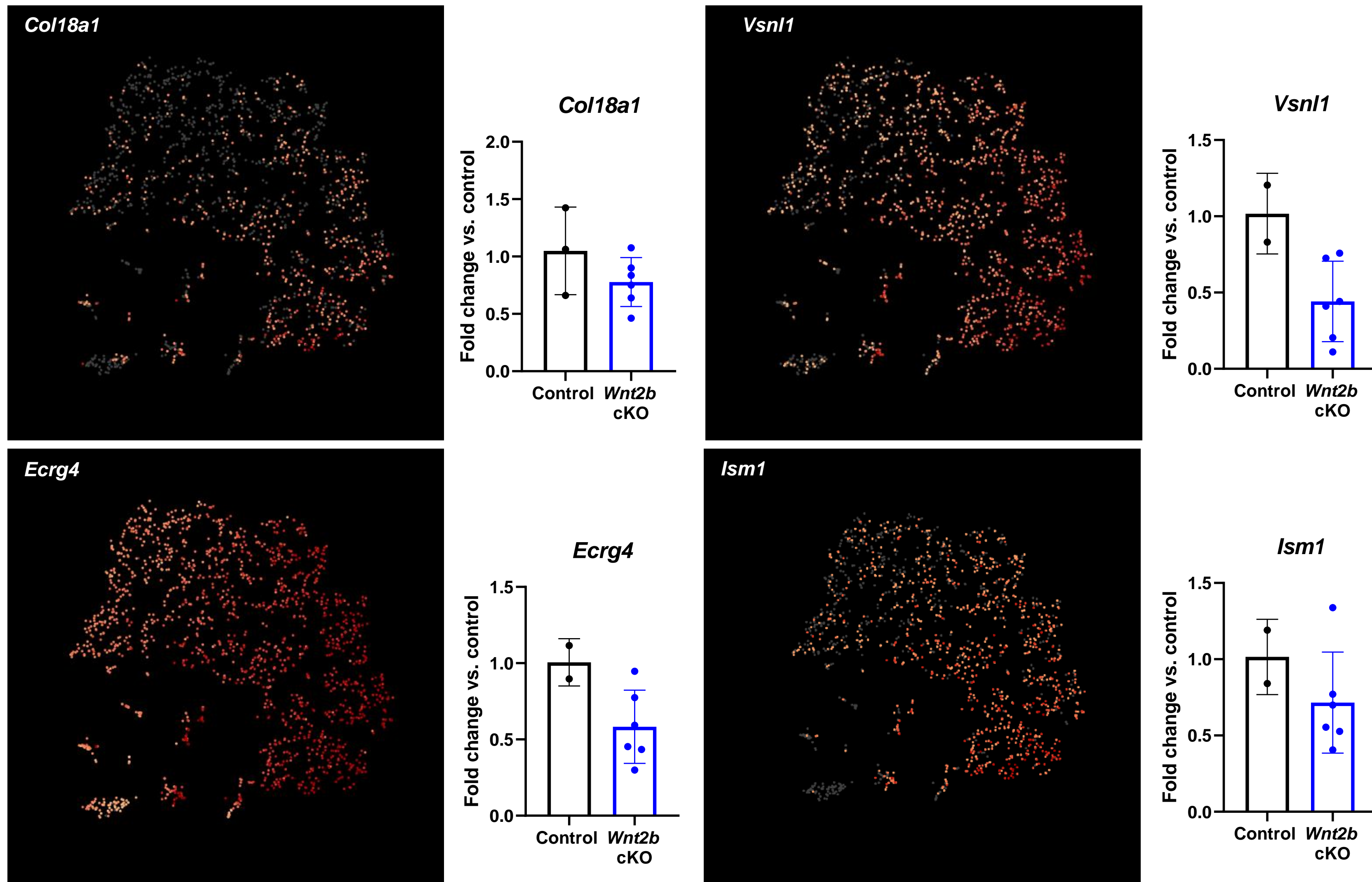
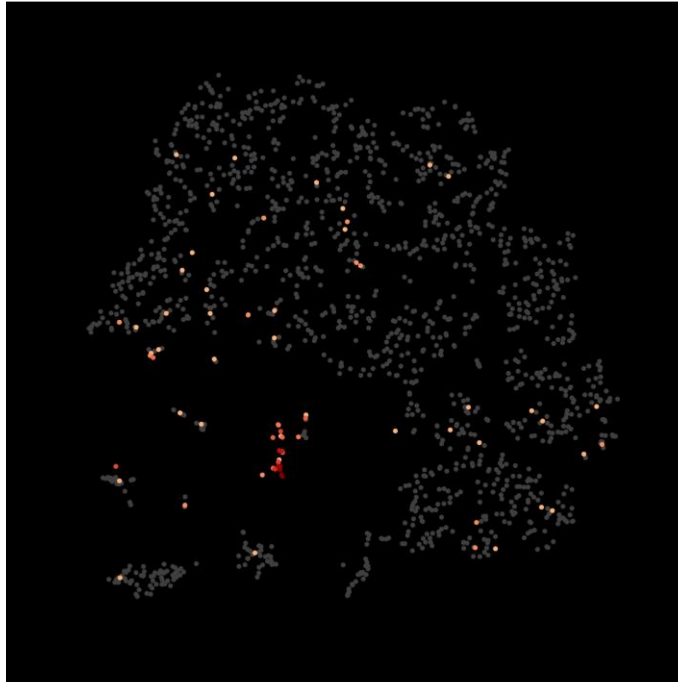


Figure 2.26. Expression patterns of Wnt-GFP genes of interest and levels *Wnt2b* cKO adrenals
tSNE plots depicting expression of genes of interest in primary Wnt-GFP cells that have not yet been characterized in the adrenal cortex. Note the zG-to-zF (right to left) expression gradient of each gene. qPCR results to the right of each plot show a downward trend in each gene expressed in *Wnt2b* cKO adrenals compared to Gli1-CreERT2-negative *Wnt2b*$^{fl/fl}$ controls.

Wnt-GFP cells to determine transcripts specific to proliferative cells of the zG. *Ki67*-positive cells were a small but mixed population of *Cyp11b2*- and *Cyp11b1*-expressing cells, and GSEA shows upregulation of proliferation markers and cancer cell modules, including E2F and DREAM target genes **(Figure 2.27)**. Our scRNAseq data provide important insights into the different transcriptional programs between undifferentiated/proliferative and differentiated/steroid-producing zG cells and provide a powerful foundation for further cell comparisons and unraveling of unique Wnt/β-catenin target gene programs.



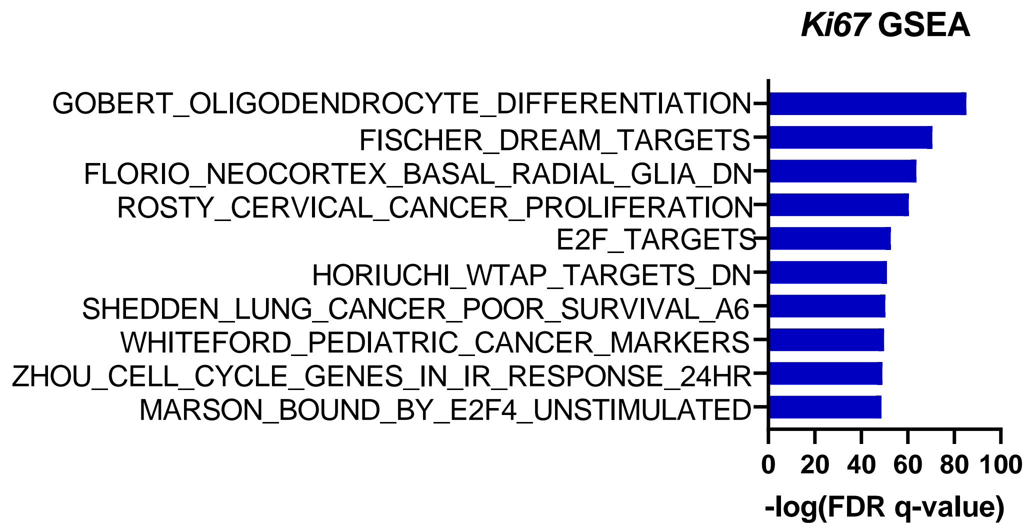


Figure 2.27. Analysis of *Ki67*-positive Wnt-GFP cells
(Top) t-SNE plot of *Ki67*-expressing Wnt-GFP cells (orange) analyzed by scRNAseq. (Bottom) GSEA on top 100 genes significantly upregulated in *Ki67*-positive adrenocortical Wnt-GFP cells.

2.11 Discussion

In this chapter, we assess the characteristics of *Wnt2b* KO mouse models in light of patients deficient in *WNT2B*. We uncovered the presence of renin-mediated aldosterone compensation in *WNT2B*-null patients who experience unalleviated hypovolemia due to chronic congenital diarrhea **(Figure 2.1; Table 2.1)**. We then found Wnt2b expression exclusively in the adrenal capsule in mice **(Figure 2.2)**. Using *Wnt2b*-floxed mice **(Figures 2.3-2.4)** from Dr. Terry Yamaguchi and in collaboration with the lab of Dr. David Breault, we generated and characterized both global and conditional *Wnt2b* KO mouse models **(Figures 2.5-2.20)**. *Wnt2b* gKO mice lack histological zG as assessed by DAB2 and β-catenin staining **(Figures 2.5-2.7)**. We show that conditional loss of Wnt2b in adult mice **(Figures 2.8-2.9)** leads to decreased adrenocortical cell proliferation and β-catenin-mediated Wnt-high signaling in the zG **(Figures 2.10-2.15)**. Dampening of Wnt-high activity in the context of *Wnt2b* loss results in decreased *Cyp11b2* expression and renin-mediated aldosterone compensation **(Figures 2.16-2.20)** as we observe in patients. Additionally, we have developed and describe here single cell RNA sequencing (scRNAseq) on primary Wnt-responsive zG cells to define unique Wnt/β-catenin-dependent transcriptional programs engaged in undifferentiated progenitor cells and differentiated aldosterone-producing cells **(Figures 2.21-2.27; Table 2.2)**.

The distinction between development and homeostasis of the adrenal cortex is of key importance in our work. As the fetal adrenal cortex develops, it sets the stage for the definitive cortex to be established, in part through the organization of the progenitor cell niche involving the capsule and zG. The role of WNT2B and the timing of its engagement,

along with the expression of Wnt/β-catenin signaling components, during development remains unaddressed in our work. However, the necessity of WNT2B in the establishment of the postnatal zG in both humans and mice cannot be understated. It is presumed to work alongside RSPO3—a potentiator of adrenocortical Wnt/β-catenin signaling also expressed in the capsule—the loss of which also results in zG depletion (Vidal et al., 2016). While *Rspo3* and *Wnt2b* expression has been shown to overlap *Gli1* expression in mesenchymal cells, it is unknown how each is transcriptionally regulated (Vidal et al., 2016; Valenta et al., 2016). Previous unpublished data from our lab from mice lacking Coup-TFII, a capsular transcription factor, suggest that it may activate *Wnt2b* expression. However, the exact co-expression pattern and transcriptional regulation of *Wnt2b* and *Rspo3* in the adrenal capsule remains unclear. Another consideration is the compensatory and redundant nature of WNT ligands observed in other tissues. *Wnt2b* is expressed alongside *Wnt6a*, *9a*, and *10a* in the adrenal capsule, although *Wnt2b* expression is markedly higher **(Appendix A)**. Given the redundancy of WNT ligand activity, it is unknown how other capsular WNT ligands may act in adrenocortical Wnt/β-catenin activity, although it is suspected that the three aforementioned capsular WNTs play a very minor role, if any.

To determine the role of WNT2B in adrenocortical development and homeostasis, we generated two *Wnt2b* KO mouse models using a previously published *Wnt2b*-floxed line (Tsukiyama and Yamaguchi, 2012). Initial studies revealed the hypomorphic nature of the *Wnt2b*-floxed allele as *Wnt2b*$^{fl/+}$ and *Wnt2b*$^{fl/fl}$ adrenals have nearly 50% and 90% reduction in *Wnt2b* expression, respectively. This hypomorphic nature is likely due to a leftover neomycin (Neo) cassette initially used to positively select for ES clones

transfected with the *Wnt2b*-floxed allele, perhaps resulting in a null transcript. While these results were unexpected, they yielded a great benefit to our work by allowing us to generate and study a Wnt2b allelic series. Indeed, Cre-negative *Wnt2b*$^{fl/fl}$ mice, harboring two functional yet hypomorphic *Wnt2b* alleles, exhibit disrupted zG zonation and morphogenesis throughout most of the adrenal cortex, although some portions exhibit normal zG histology. Cre activation exacerbates this phenotype by significantly reducing *Wnt2b* expression from an already low level. Moreover, *Wnt2b*$^{fl/+}$ mice, harboring either global *CMV-Cre* or adrenal capsule-restricted *Gli1-CreERT2*, display normal histological and molecular zonation markers. These data highlight the importance of WNT2B dosage in the adrenal cortex and suggest that the presence of one intact wild-type *Wnt2b* allele is sufficient to sustain proper zG development and homeostasis, as would be expected in heterozygous human carriers of *WNT2B* mutations who do not present with the conditions observed in their homozygous *WNT2B*-null children.

Initially, we hypothesized that WNT2B is the primary activator of adrenocortical Wnt/β-catenin signaling to maintain the undifferentiated state of zG-restricted progenitor cells. We have shown that both adrenocortical cell proliferation and *Shh* expression, two hallmarks of adrenocortical progenitor cells, are significantly decreased in *Wnt2b* cKO mice. However, the precise role of WNT2B specifically on progenitor cells remains elusive. Adrenocortical homeostasis depends upon continual self-renewal of these long-term retained progenitors. Previous studies showing that fate modulation of Wnt-responsive progenitor cells is defective in mice with Wnt/β-catenin alterations suggest that long-term loss of WNT2B would ultimately result in adrenal failure (Walczak et al., 2014; Kim, A.C. et al., 2008; Berthon et al., 2010; Vidal et al., 2016). Although both

WNT2B-null patients and mice have intact HPA axes in the contexts analyzed, lifelong depletion of self-renewing adrenocortical progenitor cells would be expected to have a dire impact on zF maintenance and function. Further studies are needed to define WNT2B-activated genes in progenitor cells involved in self-renewal versus differentiation.

Our data showing that *Wnt2b* loss results in downregulation of *Wnt4* and disruption of Wnt-high zG activity were initially surprising, although the resulting defect in RAAS engagement was to be expected. WNT4 is necessary for aldosterone production, but its exact role in the zG alongside WNT2B activity is unclear (Heikkila et al., 2004). The presence of WNT4 seems to act as reinforcement in an adrenocortical Wnt/β-catenin signaling cascade. While they are yet unknown, the unique effects of WNT2B and WNT4 on progenitor and aldosterone-producing cells, is an exciting avenue of future research, including β-catenin-mediated *Cyp11b2* transcriptional activation, cell-specific *Fzd* expression, WNT2B/4-FZD interactions, and the involvement of adrenocortical Wnt/β-catenin signaling inhibitors and other pathways, such as BMP, FGF, and SHH. We have shown extensive evidence of a capsular-cortical Shh-Wnt signaling relay, but more work must be done to define ligand engagement and transcriptional regulation more clearly at the cellular level (Finco et al., 2019). These answers may now be more easily addressed using scRNAseq and RNAScope technologies, the details of which are discussed in **Chapter 4**.

2.12 Materials and Methods

Wnt2b stable cell line Mouse parental L cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; Gibco) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). To generate a *Wnt2b* stable L cell line, Gateway cloning (Thermo Fisher) was used to transfer human *Wnt2b2*-STOP plasmid insert (Addgene #35870) into a pLenti CMV Puro DEST (Addgene #17452) destination vector. Cloned *pLenti-Wnt2b2* plasmids were transformed into DH5alpha *E. coli*, grown overnight in LB bacterial culture media with ampicillin (100 ug/mL), and isolated from bacterial cells using Plasmid Mini or Maxi Prep Kit (Qiagen) according to manufacturer's instructions. Restriction enzyme analysis followed by Sanger sequencing (Advanced Genomics Core, University of Michigan) using M13, PuroR, and WPRE primers was used to verify correct nucleotide sequence. Parental L cells were then transduced with *pLenti-Wnt2b2* lentivirus made in HEK 293T cells and selected with puromycin (7.5 ug/mL) for 7 days (Vector Core, University of Michigan). *Wnt2b2* expression was validated by qPCR using TAGGTCTTGCCTGCCTTCTG (F) and TGTCACAGATCACTCGTGCC (R) primers for human *Wnt2b2*.

Wnt CM and cell experiments L-Wnt3a and L-Wnt2b cells were grown in DMEM/F-12 (Gibco) supplemented with 20% FBS and 1% penicillin/streptomycin (P/S). For conditioned media (CM) experiments, cells were passaged at a 1:10 ratio and incubated for 2 days. After addition of fresh media, cells were grown for 5 more days to allow for

media conditioning with WNT3A and WNT2B. CM samples were then collected, sterile filtered using a 0.2 micron filter, and stored at -80°C until use. For TOPFlash assays, HEK 293 STF cells were seeded in a 24-well plate at a density of 76,000 cells/well and allowed to attach overnight in Advanced DMEM/F-12 media (Gibco) supplemented with 10% FBS, 1% P/S, and 1% GlutaMAX (Invitrogen). STF cells were incubated in L-Wnt3a/Wnt2b CM diluted in fresh HEK 293 STF cell media for 12 h. After incubation, protein lysates were collected from cells following a 15-minute incubation at room temperature in 100 uL 1X Passive Lysis Buffer (Promega). Lysates were centrifuged briefly before 20 uL was added to a white-bottom 96-well plate in triplicate for analysis. Luciferase activity was detected using Dual-Glo Luciferase Assay System (Promega) and measured on a GloMax Multi+ Detection System luminometer. For Y1 experiments, mouse Y1 cells were cultured in DMEM supplemented with 2.5% FBS, 7.5% HS, and 1% P/S. One day before experiments, Y1 cells were plated in a 6-well plate at a density of 250,000 cells/well. Cells were then incubated in untreated media or diluted (20%) L-Wnt3a/Wnt2b2 CM for 24 h. RNA was isolated from cells using RNeasy Mini Kit (Qiagen), converted to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Bio), and analyzed by qPCR (see **Table 2.2** for primer sequences).

Mice All mouse experiments were carried out in accordance with protocols approved by the Boston Children's Hospital's Institutional Animal Care and Use Committee and the University Committee on Use and Care of Animals at the University of Michigan. Mouse lines used, including *Wnt2b* fl/fl (Tsukiyama and Yamaguchi 2012), *Gli1-CreERT2* (Ahn and Joyner 2004), *CMV-Cre* (Jackson Laboratory), and *Tcf/Lef:H2B-GFP* (Ferrer-Vaquer

et al., 2010) have been described previously. Animals were maintained on a mixed background under a 12-hour light/dark cycle with *ad lib* access to food and water. To generate *Wnt2b* cKO, *Gli1-CreERT2* +/- and *Wnt2b*-floxed mice were bred and administered five doses of tamoxifen (50 mg/kg) at 6 weeks of age and sacrificed 4 weeks later. For SD experiments, mice were fed sodium deficient (0.01-0.02% NaCl) chow (Envigo) for one week.

Adrenal dissection and histology After dissection, adrenals were cleaned of periadrenal fat, weighed, and fixed in 10% neutral buffered formalin (NBF) for 24 h at room temperature. Processed adrenals were paraffin-embedded and cut in 5 micron sections for use.

qPCR Adrenals used for qPCR were dissected, cleaned of periadrenal fat, flash frozen in liquid nitrogen, and stored at -80°C until use. Frozen adrenals were added to 600 uL Buffer RLT with 2-mercaptoethanol (10 uL/mL) in sterile Lysing Matrix D tubes (MP Biomedicals) and homogenized 2 x 30 seconds using a Bead Bug Homogenizer. RNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions and eluted in 20 uL (male) or 30 uL (female) nuclease-free water. 500 ng RNA was treated with DNase (Invitrogen) and converted to cDNA using cDNA High Capacity Reverse Transcription Kit (Applied Bio) according to manufacturer's instructions. 10 ng cDNA was analyzed by qPCR using Power SYBR Green PCR Master Mix (Invitrogen) on a QuantStudio 3 thermocycler. Primers are listed in **Table 2.3**.

Table 2.3. Primer sequences used for genotyping and qPCR*

Target	Forward (5'-3')	Reverse (5'-3')
Gli1-CreERT2	GGGATCTGTGCCTGAAACTG	CTTGTGGTGGAGTCATTGGA (WT) CAGGTTCTTGCGAACCTCAT (Mut)
Cre	CCCGCAGAACCTGAAGATGT	GTTCGAACGCTAGAGCCTGTTT
Il3 (*Cre* int control)	GGGACTCCAAGCTTCAATCA	TGGAGGAGGAAGAAAAGCAA
Gfp	GCACGACTTCTTCAAGTCCGCCATGCC	GCGGATCTTGAAGTTCACCTTGATGCC
Fabpi (Gfp int control)	CCTCCGGAGAGCAGCGATTAAAAGTGTCAG	TAGAGCTTTGCCACATCACAGGTCATTCAG
m*Wnt2b*	CATGCTCAGAAGCAGCCGGG	GTTGATCATGGTGCCGACCG
m*Wnt4*†	CCCTGTCTTTGGGAAGGTGGTG	CACCTGCTGAAGAGATGGCGTATAC
m*Axin2*†	TGGGGAGTAAGAAACAGCTCC	CCAGCTCCAGTTTCAGTTTCTC
m*Cyp11b2*§	GCACCAGGTGGAGAGTATGC	CCATTCTGGCCCATTTAGC
m*Shh*§	ACCCCGACATCATATTTAAGGA	TTAACTTGTCTTTGCACCTCTGA
m*Hsd3b6*	ATCTGGAGGAGATCAGGGTCC	ACAGCTGCAGTGTGGATAAC
m*Vsnl1*	TCCAGCAGCTCTATGTGAAGTT	ATGGTGCCATCACCGTTCTT
m*Ism1*	CGTCCAAACTGCCCAGGAAT	AGCAGACTCACTTCAGTGGC
m*Col18a1*-1	CTGGCATTGGCTATGAGGGT	GATCCTCACCTGCCCAGCA
m*Col18a1*-2	CTGGGCAGGTGAGGATCTGG	CCTCCAGCAGCACCTTCC
m*Ecrg4*	GCCTGGGTCCAGATGGCATA	TGTCGTTTGGCACGCTTCAG
m*Beta-actin*	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC
h*Wnt2b2*	TAGGTCTTGCCTGCCTTCTG	TGTCACAGATCACTCGTGCC

*Primer stocks were stored at 100 uM concentrations at -80°C. Working primer stocks were diluted 1:10 (10 uM) and stored at -20°C. Primers were used at a concentration of 500 nM for all genotyping and qPCR protocols. All primers were developed using Primer Blast (NCBI) in lab unless noted below.

†Developed by Drelon et al., 2016a

§Developed by Vidal et al., 2016.

Immunohistochemistry Adrenal sections were deparaffinized (HistoClear), rehydrated, and boiled in appropriate antigen retrieval buffer +0.05% Tween for 20 minutes followed by 20 minutes of cooling at room temperature. Sections were then blocked in 2.5% normal horse serum for 1 h at room temperature and incubated overnight at 4°C with primary antibodies for Ki67 (1:200), cleaved caspase 3 (1:200), and active β-catenin (1:500) (see **Table 2.4**). Following primary antibody incubation, antigens were detected using ImmPRESS Excel polymer kit (Vector Laboratories) according to manufacturer's instructions. Primary antibodies were observed using DAB-based detection and imaged on a Nikon E800 brightfield microscope.

Immunofluorescence with TSA amplification For immunofluorescent staining, adrenal sections were prepared according to standard protocol used for IHC (above). Non-specific antibody binding was blocked using 2.5% horse serum + 1% BSA. Following primary antibody incubation, sections were prepared and visualized using HRP-polymer detection (Vector Laboratories) and Alexa fluor tyramide signal amplification (TSA) (Thermo Fisher) according to manufacturer's instructions. Briefly, sections were incubated in species-specific polymer for 30 minutes at room temperature followed by incubation in 0.3% hydrogen peroxide at room temperature for 20 minutes to inhibit peroxidase activity. Primary antibodies (see **Table 2.3**) were detected using Alexa fluor TSA reagent (1:100). For co-staining, sections were incubated in 0.02% HCl to clear any unbound polymer before proceeding. DNA was detected by a 10 minute incubation in Hoechst (1:10,000 in PBS) at room temperature. Sections were mounted in ProLong Gold, cured overnight,

and imaged using an X-Cite Series 120 Q fluorescent lamp on a Nikon Opti-Phot 2 microscope.

Table 2.4. Antibodies used for IHC

Antigen	Developer	Species	Dilution	Antigen Retrieval	Blocking
DAB2	Upstate Biotech #06-431	Mouse	1:500	Sodium citrate, pH6.0	M.O.M. blocking reagent
AKR1B7	Santa Cruz, sc-27763	Goat	1:200	Sodium citrate, pH6.0	2.5% NHS
β-catenin	BD Biosciences, 610153	Rabbit	1:500	Sodium citrate, pH6.0	1X PBS + 5% NGS
LEF1	Abcam, ab137872	Rabbit	1:400	1M Tris-EDTA, pH 9.0	1X PBS + 1% BSA
Ki67	BD Pharmingen, 550609	Rabbit	1:200	Sodium citrate, pH6.0	2.5% NHS
Cleaved caspase 3	Cell Signaling, 9664S	Rabbit	1:200	Sodium citrate, pH6.0	2.5% NHS
Active β-catenin	Cell Signaling, 8814	Rabbit	1:500	Sodium citrate, pH6.0	2.5% NHS
GFP	Aves, GFP-1020	Goat	1:1000	Sodium citrate, pH6.0	2.5% BHS + 1% BSA
CYP11B2	Celso Gomez-Sanchez	Rabbit	1:500	Sodium citrate, pH6.0	2.5% NHS + 1% BSA

Single molecule ISH and quantification Adrenal sections were prepared as above and used within one week of sectioning. Single molecule ISH was performed using RNAScope Brown Detection Kit (Advanced Cell Diagnostics) according to manufacturer's instructions and as previously reported (Basham et al., 2019). Antigen retrieval boiling time of 7 minutes was previously determined and used for adrenal sections. Images were obtained on a Nikon E800 microscope and subsequently processed to quantify transcripts using threshold adjustments in ImageJ.

Steroid hormone measurements Mouse trunk blood samples were collected between 9:00 and 10:00 am by decapitation within 30 seconds of handling to minimize acute stress response. Blood was collected in sodium heparin-coated evacuated tubes (Fisher Scientific) and centrifuged at 1800 x g for 15 minutes at 4°C to separate plasma. 75 uL

plasma aliquots were separated and stored at -80°C until analysis. Plasma aldosterone was measured by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) performed by the lab of Dr. Adina Turcu at the University of Michigan.

Mouse renin assays Mouse renin levels were determined using TaqMan-based qPCR and ELISA. For qPCR, the upper left kidney quadrant of mice was dissected and flash-frozen in liquid nitrogen. Following RNA isolation (RNeasy Kit, Qiagen) and reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Bio), mouse Ren1 (Mm02342887_mH) and β-actin (Mm02619580_g1) TaqMan probes with TaqMan Fast Advanced Master Mix (Thermo Fisher) were used to quantify Ren1 in 10 ng of kidney cDNA. For plasma renin, trunk blood was collected as previously described and stored at -80°C until analysis. Samples were thawed and analyzed using Mouse Renin ELISA Kit (Thermo Fisher) according to manufacturer's instructions. Plasma samples were diluted at 1:15 for use as previously described (Taylor et al., 2020).

Single cell RNAseq Adrenals from wild-type and Tcf/Lef:H2B-GFP (Wnt-GFP) female mice from 8-12 weeks of age were dissected and weighed in wash buffer (DMEM/F-12 + 5% FBS + 5 ug/mL insulin + 50 ug/mL gentamicin). Adrenals were then minced on a clean 10 cm Petri dish using sterile razor blades, and the homogenized tissue was transferred to fresh collagenase/DNase solution (DMEM/F-12 + 5% FBS + 5 ug/mL insulin + 50 ug/mL gentamicin + 1 mg/mL collagenase + 0.1 mg/mL DNase I). Samples were incubated in a humidified chamber at 37°C for up to one hour and pipetted up and down every 10 minutes to obtain single cells. After dissociation, cells were sterile filtered

through a 70 micron filter and diluted with HBSS to inactivate enzymes. Samples were then centrifuged at 1500 rpm for 10 minutes at 4°C, washed twice with HBSS + 2% FBS, and resuspended at approximately 1x10^6 cells/mL. To obtain live Wnt-GFP cells, both WT (GFP-) and Wnt-GFP cells were incubated with Ruby (Thermo Fisher) and DAPI and analyzed by fluorescence activated cell sorting (FACS). Live Wnt-GFP cells (Ruby+; DAPI-; GFP+) were collected, counted, and analyzed by single cell RNA sequencing (scRNAseq) using 10X Genomics Chromium platform (Advanced Genomics Core, University of Michigan). All analyses performed in this thesis were done using Loupe Cell Browser and GSEA online tool (UC San Diego/Broad Institute; https://www.gsea-msigdb.org/gsea/index.jsp).

CHAPTER 3 *Ccdc80* is a Novel Wnt/β-catenin Target Gene

3.1 Introduction

Wnt/β-catenin signaling drives both developmental and homeostatic transcriptional programs in various tissues. Target gene expression activated by non-phosphorylated β-catenin in WNT ligand-responsive cells can maintain stem/progenitor cell states, induce proliferation or differentiation, and/or stimulate the function of differentiated cells, depending on the tissue and context of signaling. As Wnt/β-catenin signaling is essential for adrenocortical development, homeostasis, and function, we sought to identify β-catenin-dependent target genes that mediate these processes (Berthon et al., 2010; Kim, A.C. et al., 2008). Expression of classic Wnt/β-catenin target genes, such as *Axin2* and *Lef1*, has been observed in the adrenal cortex for decades (Filali et al., 2002; Jho et al., 2002). However, other adrenal cortex-specific Wnt/β-catenin target genes have remained largely uncharacterized until recently. A previous thesis project in our lab utilizing fluorescence-activated cell sorting (FACS) and RNA sequencing analysis discovered *Ccdc80* expression in Wnt-responsive adrenocortical cells, providing a new lead in adrenocortical Wnt/β-catenin activity (Walczak et al., 2014). In this chapter, I will summarize the roles of CCDC80 in various tissues, detail our experiments to help define its role in the adrenocortical biology, and describe future experiments to be done and their implications in adrenocortical Wnt signaling.

Coiled-coil domain-containing protein 80 (CCDC80) is a secreted glycoprotein that is conserved across many vertebrate species, including rat, mouse, chicken, and human. *Ccdc80* was first discovered in rat and named *steroid-sensitive gene 1* (*Ssg1*) as its expression was regulated by androgen and estrogen in rat prostate glands and mammary and uterine tissues, respectively (Marcantonio et al., 2001a; Marcantonio et al., 2001b). The mouse homolog of *Ccdc80*, coined *upregulated in Brs3-deficient mice* (*Urb*), was discovered in mice lacking bombesin receptor subtype-3 in which brown adipose tissue expressed significantly higher levels of *Urb*/*Ccdc80*, suggesting a role in adipogenesis and metabolism (Aoki et al., 2002). Indeed, studies from additional groups have shown that downregulation of *Ccdc80* is associated with obesity and metabolic disease in mouse and human, further supporting an essential role in normal adipocyte differentiation and glucose homeostasis (Okada et al., 2008; Tremblay et al., 2009; Tremblay et al., 2012; Grill et al., 2017; Osorio-Conles et al., 2017; L. Liu et al., 2020; Wang et al., 2021).

Several studies have also defined the role of equarin, the *Ccdc80* homolog expressed in chicken, in eye development. Equarin is expressed in the lens transition zone, where lens cell differentiation occurs, in the chick eye, suggesting that equarin plays an important role in cell differentiation (Mu et al., 2003). The authors then showed that exogenous equarin transcripts injected into the lens of developing *Xenopus* embryos resulted in stunted eye development, likely due to increased cell adhesion that impaired proper cell movement. Further studies expanded upon the role of equarin in cell adhesion in showing its interactions with heparin sulfate proteoglycans (HSPGs)—transmembrane proteins involved in extracellular matrix (ECM) interactions and growth factor signaling—and fibroblast growth factor (FGF) (Song et al., 2012; 2013). FGF-dependent lens

differentiation was significantly decreased in equarin-deficient chick lenses (Song et al., 2012). Conversely, overexpression of equarin in the developing chick eye resulted in remarkable changes in F-actin expression pattern, cytoskeleton organization, and an

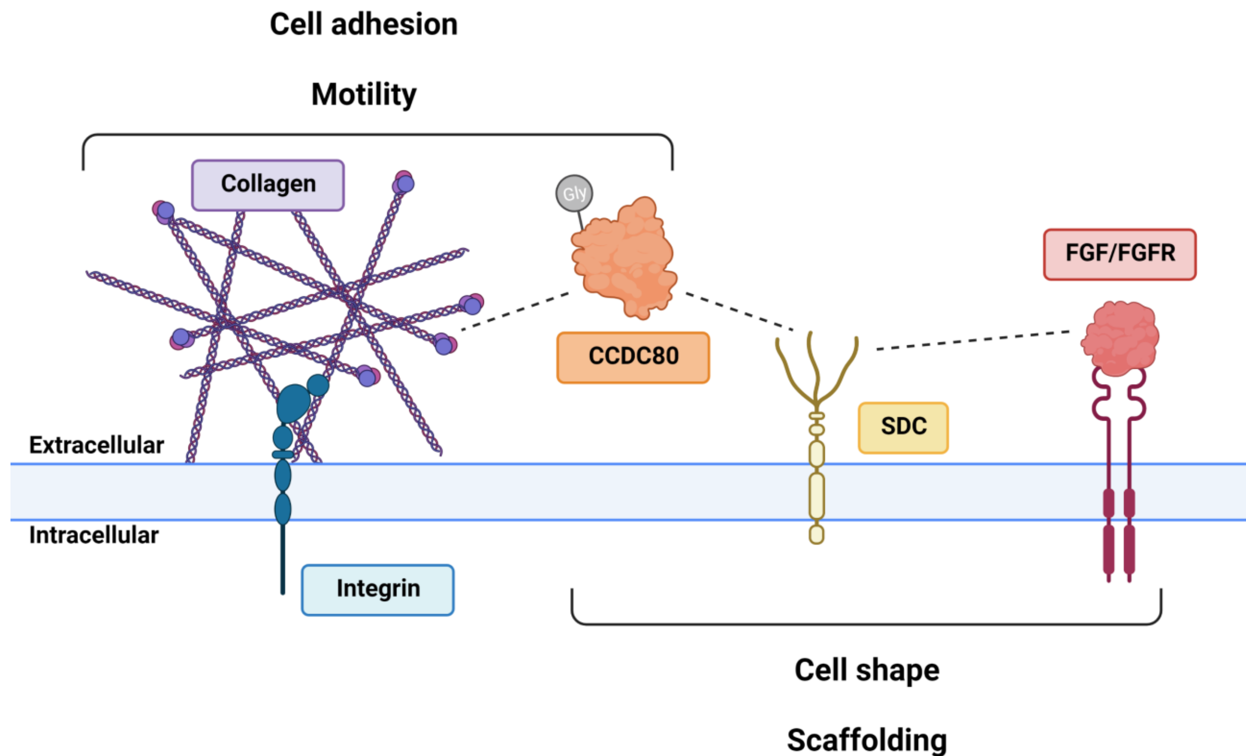


Figure 3.1. Schematic of CCDC80 interactions
CCDC80 (orange, middle) is a glycosylated (Gly) ligand that interacts with several proteins of the extracellular matrix (ECM). (Left) CCDC80 binds to collagens, prominent structural ECM proteins that interact with integrins on the surface of the cell membrane. These interactions then regulate cell adhesion and motility and can also activate several intracellular signaling pathways. (Right) CCDC80 binds to both syndecan (SDC, gold) and fibroblast growth factor (FGF, red), which has potential intracellular effects on cell shape and scaffolding through cytoskeletal rearrangements.

abnormal lens structure (Song et al., 2013). Together, these studies have defined a role for CCDC80 in ECM structure, HSPG binding, and FGF signaling **(Figure 3.1)**.

While these and other models have highlighted the role of CCDC80 in cell differentiation and adhesion, several studies have also indicated *Ccdc80* as a tumor suppressor gene in various human cancers. In fact, the human *Ccdc80* homolog was first named *DRO1*, signifying its being *downregulated by oncogenes* in colon and pancreatic

cancer cell lines expressing constitutively active β-catenin or K-ras (Bommer et al., 2005). The addition of *DRO1* mRNA to transformed cell lines significantly attenuated growth and led to anchorage-dependent apoptosis, the first evidence of a substantial effect of CCDC80 in human cancer. A similar tumor suppressor role, among others, for *Ccdc80* was later described in thyroid, ovarian, and other pancreatic and colorectal cancer (CRC) cell lines and mouse models (Ferraro et al., 2013; Leone et al., 2015; Paullin et al., 2017; Zheng et al., 2020; Hong et al., 2020; Grill et al., 2014; Grill et al., 2018; Pan et al., 2019; Wang et al., 2020).

The downregulation of *Ccdc80* in CRC models raises a curious yet important question about the relation between *Ccdc80* expression and Wnt/β-catenin signaling, which is activated in most CRCs due to *APC* loss (Rowan et al., 2000; Schell et al., 2016). One study showed that shRNA knockdown of *CCDC80* in 3T3-L1 adipocytes led to a significant increase in TCF/LEF-mediated TOPFlash reporter activity and *Axin2* expression (Tremblay et al., 2009). This led to the hypothesis that CCDC80 negatively regulates Wnt/β-catenin signaling. *Ccdc80* has been noted as a Wnt/β-catenin target gene in human pancreatic cells and mouse hepatocytes and CRC cells, potentially to regulate Wnt/β-catenin activation in a negative feedback loop (Wan et al., 2012; Chan et al., 2019; Pan et al., 2019). Notably, our lab generated data in both a Wnt-activated mouse ACC cell line and primary GFP-labeled Wnt-responsive adrenocortical cells that supported *Ccdc80* as a novel Wnt/β-catenin target gene in the adrenal cortex (Walczak et al., 2014).

Our lab was the first to show subcapsular *Ccdc80* expression in mouse adrenocortical zG cells by *in situ* hybridization, validating it as a putative Wnt-responsive

gene in adrenocortical progenitor cell biology (Walczak et al., 2014). *Ccdc80* was upregulated in mouse ATC7L cells transiently expressing constitutively active β-catenin (β-catS33Y) or treated with BIO, a drug that stabilizes β-catenin to enhance target gene activation. These data have since been validated using single cell RNA sequencing (scRNAseq) on primary GFP-labeled Wnt-responsive (Wnt-GFP) adrenocortical cells from mice, which shows *Ccdc80* expression in about 14% of the total sorted cells (230 of 1,551) **(Figure 3.2)**. Additionally, conditioned media (CM) collected from CCDC80-expressing cells suppressed corticosterone secretion and expression of zF steroidogenic genes *StAR*, *Cyp11a1*, and *Cyp11b1* (Walczak et al., 2014). Together, these results suggested that *Ccdc80* is a zG-restricted Wnt/β-catenin target gene that encodes a ligand involved in the suppression of zF-specific steroidogenesis through a non-cell autonomous mechanism.

While the necessity of Wnt/β-catenin signaling for proper adrenocortical development and function is well known, its full scope in adrenocortical biology is far from understood. Novel adrenocortical Wnt/β-catenin target genes therefore provide opportunity for critical new insights into Wnt/β-catenin signaling in the adrenal development, zonation, structure, and steroidogenic capacity. Furthermore, as activating Wnt/β-catenin signaling alterations are present in 40% of ACCs, novel β-catenin targets also provide new therapeutic targets for Wnt-active cases that may lead to more effective treatment options (Zheng et al., 2016). Experiments from our lab showing *Ccdc80* expression is unique to Wnt-responsive adrenocortical zG cells and that it suppresses glucocorticoid production provided a promising foundation to studying adrenocortical CCDC80 *in vivo*.

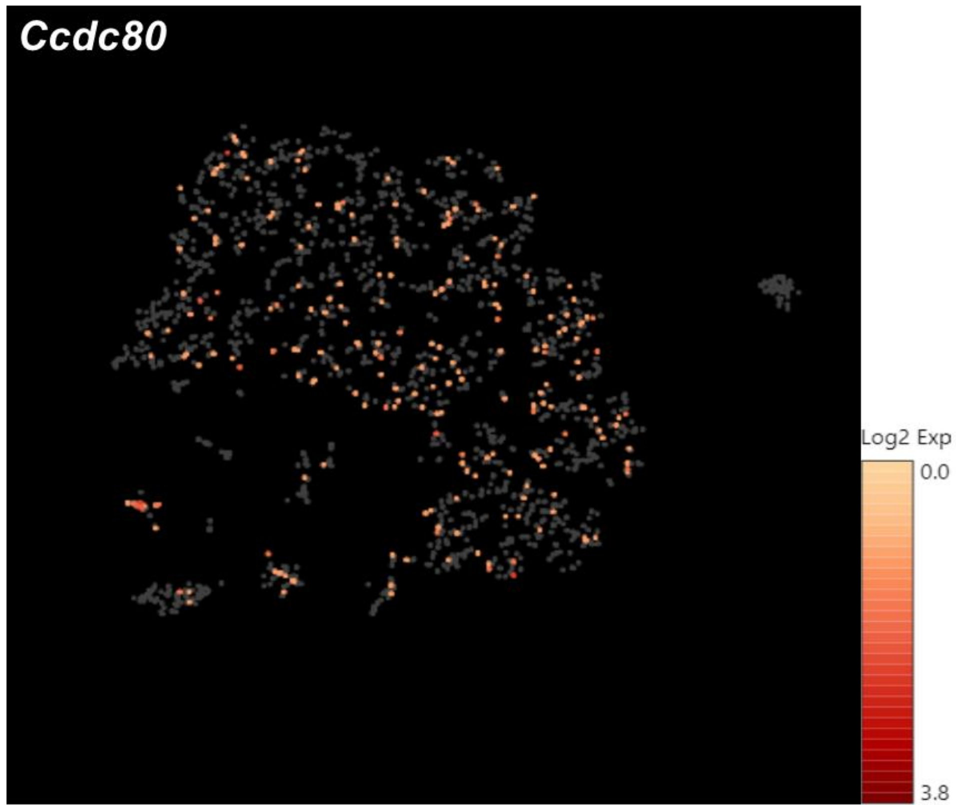


Figure 3.2. *Ccdc80* is expressed in Wnt-responsive adrenocortical cells
tSNE plot depicting *Ccdc80* expression in the mouse adrenal cortex. GFP-labeled Wnt-responsive (Wnt-GFP) cells of the adrenal cortex were sorted and analyzed by single cell RNA sequencing (scRNAseq). Each dot represents a single cell. *Ccdc80*-expressing cells (orange) represent only 14% of the sorted Wnt-GFP population. Cells not expressing *Ccdc80* are shown in grey.

In addition, these results offered a new avenue to studying the adrenocortical ECM, about which little is known. The ECM provides extracellular protein scaffolding essential for cell attachment and tissue structure as well as multiple cell signaling mechanisms, thus creating a network of fibrous proteins, growth factors, transmembrane receptors, and various other biological and biochemical components making up the cellular microenvironment (reviewed in Hynes 2009; Hynes and Naba 2012). The adrenocortical ECM is made up of collagen IV, laminin, and fibronectin, the former being expressed throughout the cortex while the latter two display zonal expression patterns (Kikuta et al., 1991; Chamoux et al., 2001; Campbell et al., 2003). Interestingly, while collagen IV was found to enhance cortisol production in primary human cell cultures, laminin inhibits this effect and instead promotes cell proliferation, a key characteristic of the adrenocortical progenitor cell niche (Chamoux et al., 2002; Otis et al., 2007). **Therefore, we hypothesized that CCDC80 helps to maintain the ECM of the adrenocortical progenitor cell niche, a tightly regulated microenvironment containing Wnt-responsive progenitor cells.** It is worthy also to note that Wnt/β-catenin signaling itself plays a fundamental role in ECM biology in several tissues (Hamburg-Shields et al., 2015; Dzobo et al., 2015; Enzo et al., 2015). With these results in view, Ccdc80 quickly became an exciting link between Wnt/β-catenin signaling and ECM maintenance in the adrenal cortex.

3.2 *Ccdc80*-null mice have encapsulated adrenocortical nodules early in life

Given its primary role in promoting cell adhesion through ECM interactions, we hypothesized that CCDC80 loss would disrupt the adrenocortical ECM, particularly in the zG. To test this hypothesis, we obtained whole-body *Ccdc80* knockout (KO) mice from the lab of Dr. Frank Kolligs (Grill et al., 2014). Surprisingly, *Ccdc80* KO adrenals exhibit marked disorganization of mesenchymal cells expressing COUP-TFII. Whereas COUP-TFII-expressing cells normally reside only in the adrenal capsule, these cells are present within the adrenal cortex of *Ccdc80* KO mice and encapsulate small nodules composed of steroidogenic SF1-expressing cells **(Figure 3.3, top)**. SF1-expressing cells adjacent to COUP-TFII-expressing capsule cells of the encapsulated nodules also express CYP11B2, while cells of the inner nodule express CYP11B1 **(Figure 3.3, bottom)**. These data suggest that CCDC80 plays an essential role in promoting cell adhesion in the adrenal capsule, possibly through HSPG binding and/or FGF signaling modulation. Additionally, the observed adrenal nodules displayed steroidogenic enzyme expression patterns typical of the adrenal cortex, suggesting that they have *bona fide* steroidogenic capacity.

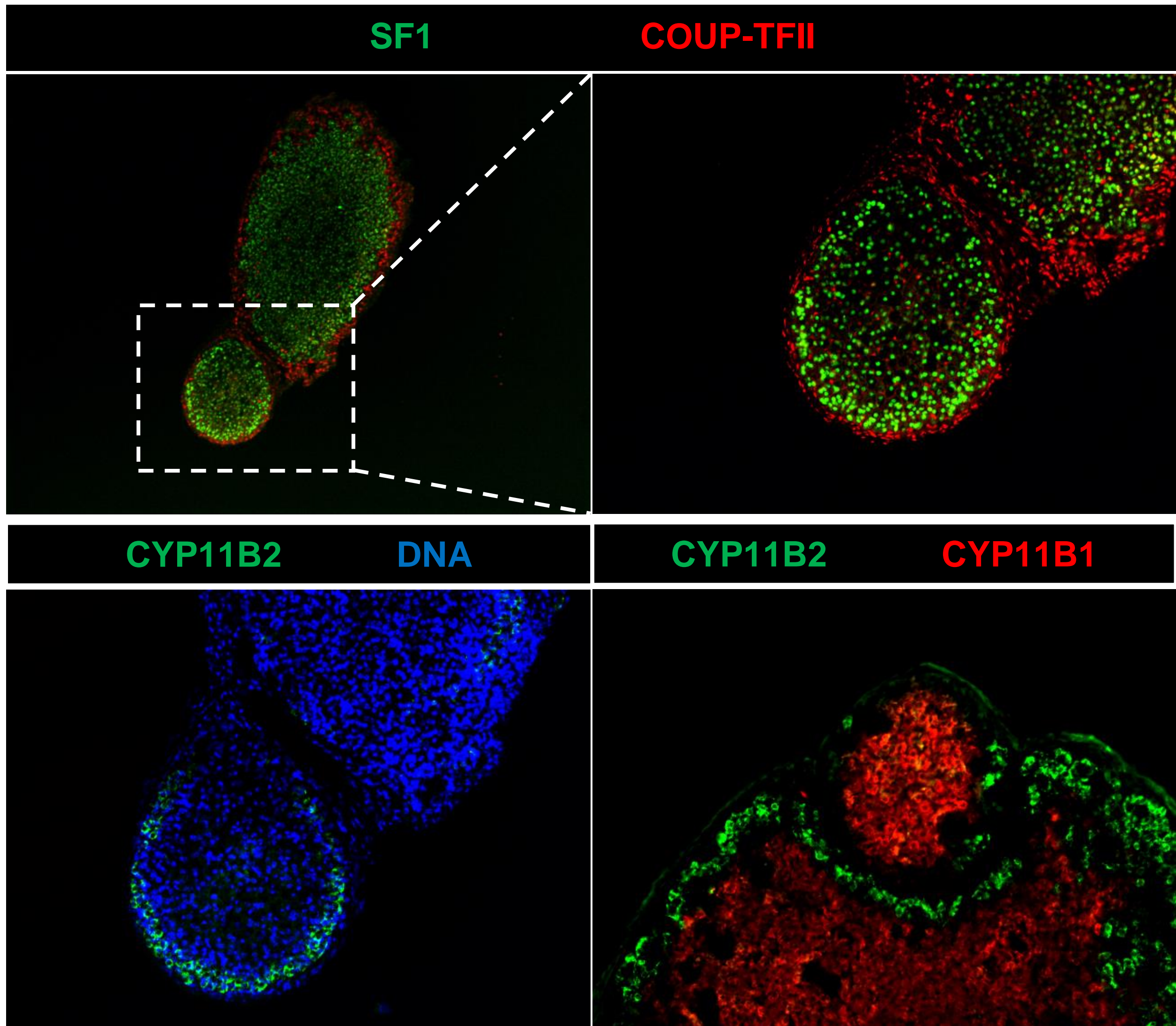


Figure 3.3. ***Ccdc80*** **KO mice develop adrenocortical nodules early in life**
Immunofluorescent (IF) staining of adrenal sections from *Ccdc80* KO mice. (Top) Images of adrenocortical nodules composed of SF1-expressing cells (green) encapsulated by mesenchymal capsule cells expressing COUP-TFII (red) Enlarged image of nodule contained in white box is shown on the top right. (Bottom) IF images from two different *Ccdc80* KO mice showing expression of aldosterone synthase (CYP11B2; green) in the subcapsular region and zF marker CYP11B1 (red) in the inner region of an encapsulated nodule.

Since the capsular defect in *Ccdc80* KO mice was observed early in life, I analyzed mice at 6, 9, and 12 months of age. However, nodules were only rarely found, and the adrenal capsule and cortex appeared normal as observed by histology **(Figure 3.4)**.

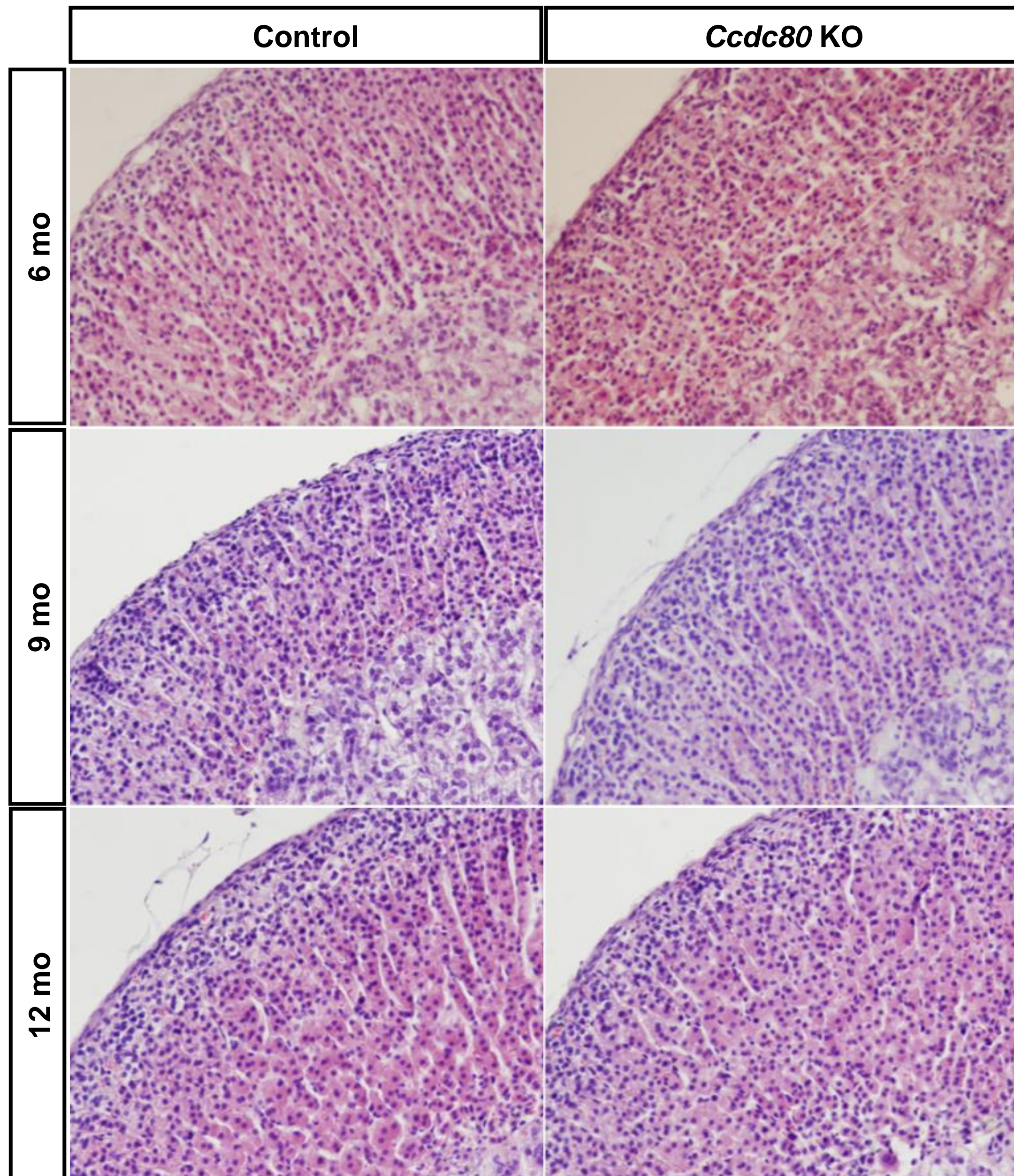


Figure 3.4. Histological images of adrenals from *Ccdc80* control and KO mice
Adrenals from *Ccdc80* control (left) and KO (right) mice were dissected at 6 (top), 9 (middle), and 12 (bottom) months of age. Hematoxylin and eosin (H&E) staining revealed no morphological defect in the capsule or subcapsular cortex.

3.3 Effect of CCDC80 on Y1 cell migration

Given its role in promoting cell adhesion, we hypothesized that mouse Y1 cell migration would be restricted in the presence of CCDC80. To test this hypothesis, I performed scratch assays on semi-confluent Y1 cells transfected with either an empty vector or *Ccdc80-HA-myc*. Images obtained 24, 48, and 72 hours post-scratch (hps) indicate that *Ccdc80* transfection has little to no impact on Y1 cell migration and gap closure **(Figure 3.5)**.

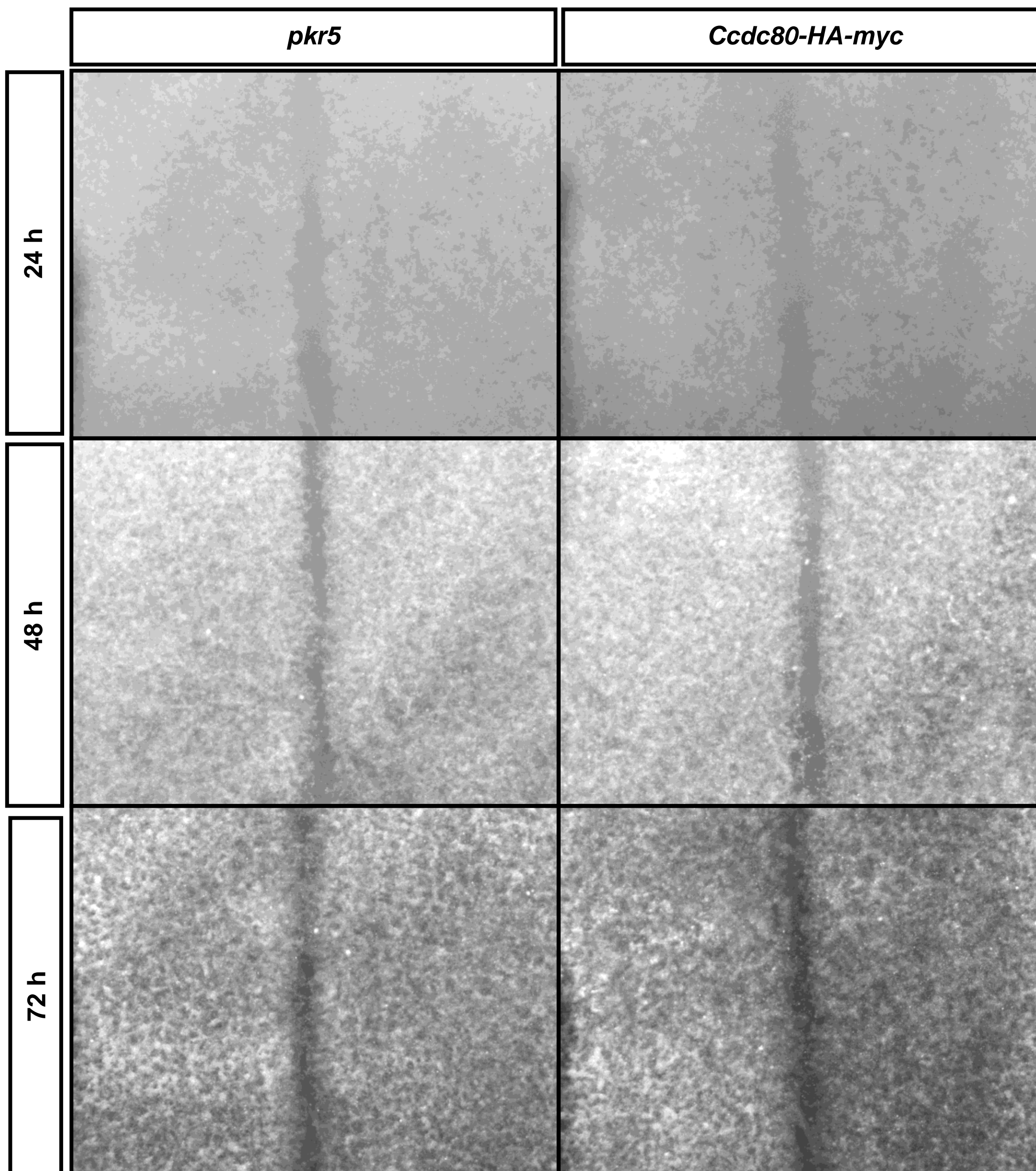


Figure 3.5. Effect of CCDC80 on Y1 adrenocortical cell migration
Representative images of scratch assays performed on mouse Y1 cells transfected with either empty vector (*pkr5*, left) or *Ccdc80-HA-myc* (right). Cell migration was assessed using brightfield microscopy at 24 (top), 48 (middle), and 72 (bottom) hours post-scratch. Images show no clear restriction on the migration of Y1 cells transfected with *Ccdc80* compared to empty vector controls.

3.4 CCDC80 interacts directly with heparin sulfate chains *in vitro*

The observation of adrenocortical nodules fully encapsulated by COUP-TFII cells in *Ccdc80* KO mice led to the hypothesis that CCDC80 mediates cytoskeletal organization and ECM structure of the progenitor cell niche through HSPG binding and/or FGF signaling. Previous studies have shown that the main HSPG binding partner of CCDC80 is syndecan 3 (SDC3) (Song et al., 2012). To test for a CCDC80-SDC3 interaction, I first investigated the capacity of tagged CCDC80 (CCDC80-HA-myc) protein to interact with heparin sulfate (HS) beads *in vitro*. Western blot verified the expression of CCDC80-HA-myc protein in transfected HEK293T cells **(Figure 3.6)**. Whole cell lysates and CM from HEK293T cells expressing CCDC80-HA-myc were incubated overnight with HS beads. Immunoprecipitation using anti-HA antibody and both whole cell protein lysates and CM shows a strong interaction between CCDC80 and HS chains as expected **(Figure 3.7)**.

Therefore, I then assessed a CCDC80-SDC3 interaction by co-immunoprecipitation (co-IP) in HEK293T cells transiently transfected with *Ccdc80-HA-myc*. While both proteins can be immunoprecipitated successfully, no interaction between

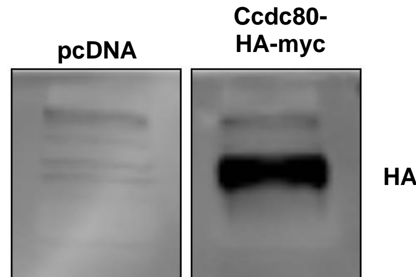


Figure 3.6. CCDC80-HA-myc expression by transient transfection
HEK293T cells were transiently transfected with with pcDNA (empty vector) or Ccdc80-HA-myc. Protein lysates were harvested 48 h later and analyzed by Western blot. Anti-HA immunoblotting shows high expression of the tagged CCDC80 protein (right). Tagged Ccdc80 construct was a generous gift from the Carter-Su lab (O'Leary et al., 2013

CCDC80 and SDC3 was observed by co-IP and Western blot (data not shown). These results suggest that, while the tagged CCDC80 protein binds strongly to HS chains, its interaction with SDC3 *in vitro* may be weak or transient in the experiments performed. Further experiments with endogenously expressed CCDC80 and SDC3, along with other SDCs, using adrenal lysates may reveal protein-protein interactions of interest that are not observed in the experiments outlined here.

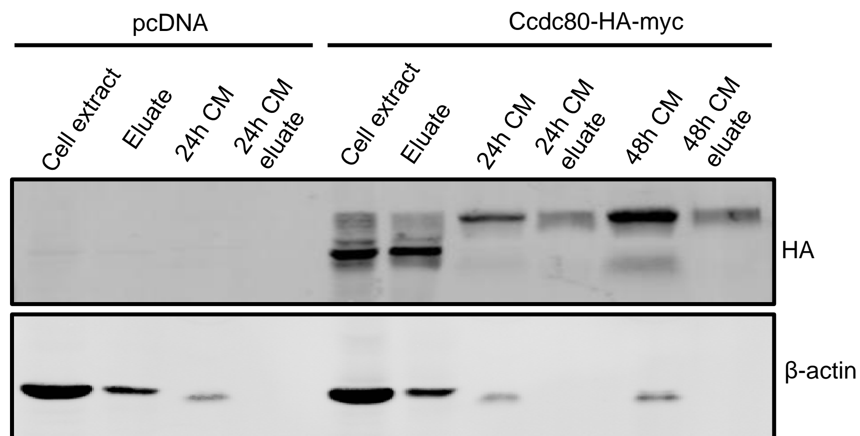


Figure 3.7. CCDC80 interacts strongly with heparin sulfate *in vitro*
Cell lysates and CM samples collected from HEK293T cells transfected with pcDNA (empty vector; left) or Ccdc80-HA-myc (right) were incubated overnight with heparin sulfate (HS) beads. Western blots for HA and β-actin shown here confirm the absence of CCDC80 protein in empty vector controls. CCDC80 was successfully immunoprecipitated from both cell lysates and CM using HS beads, indicating a strong interaction. IP eluates show moderate loss of CCDC80 throughout the protocol. Note the increased size of CCDC80 from CM samples, likely due to glycosylation. β-actin is shown as a protein loading control with which CM samples are minorly contaminated.

3.5 Discussion

In this chapter, I review the relevant literature on the role of the Wnt/β-catenin target gene coiled-coil domain-containing 80 (CCDC80) in extracellular matrix (ECM) biology, including its interactions with heparin sulfate proteoglycans (HSPGs) and FGF **(Figure 3.1)**. *Ccdc80* expression previously reported in Wnt-responsive adrenocortical cells (Walczak et al., 2014) was validated using scRNAseq **(Figure 3.2)**. I characterize the basic adrenal phenotype observed in *Ccdc80* KO mice, which have adrenocortical nodules encapsulated by mesenchymal capsule cells early in life **(Figure 3.3)**. However, nodules were not found in older mouse adrenals, and the cortex and capsule appear histologically normal overall **(Figure 3.4)**. To further explore the role of CCDC80 in adrenocortical biology, I developed *in vitro* systems to assess the effect of CCDC80 on adrenocortical cell migration and to verify the interaction of tagged CCDC80 with heparin sulfate chains, but a direct interaction with predicted HSPG binding partner Syndecan 3 (SDC3) was not observed **(Figure 3.5-3.7)**. Together, while these data are not definitive in identifying the role of CCDC80 in adrenocortical biology, they provide initial insights into its potential effect on adhesion of cells in the capsule and/or cortex and establish both *in vitro* and *in vivo* models to further define its role as an adrenocortical Wnt/β-catenin target gene.

How CCDC80 participates in adrenocortical ECM remodeling and steroidogenesis are two exciting areas warranting further study. While little is known about the adrenocortical ECM, several bodies of literature provide foundational premises to hypothesize that CCDC80 is an essential player. First, CCDC80 is known to bind to several ECM-related proteins, including collagens and syndecans, as well as FGF

(Sasagawa et al., 2016; Song et al., 2013; O'Leary et al., 2013). Secondly, several studies in mice and chick embryos show that CCDC80 promotes cell adhesion both *in vitro* and *in vivo* (Song et al., 2013; Song, Tanaka, and Ohta 2014). Finally, as *Ccdc80* has been identified as a Wnt/β-catenin target gene in different cells and tissues, its expression in Wnt-responsive cells of the adrenal cortex, which includes adrenocortical progenitor cells, further supports the ligand as an ECM component in the Wnt-responsive zG (Walczak et al., 2014).

Whether CCDC80 is necessary for the development or maintenance of either the capsular or subcapsular ECM is a major unanswered question. While *Ccdc80* is expressed primarily in the zG, it is unknown how far the ligand can diffuse and on which cell population(s) it may be acting. Our data showing encapsulated adrenocortical nodules suggest either that CCDC80 diffuses to the ECM of mesenchymal capsule cells to promote cell adhesion, or that it acts in a cell-autonomous fashion to maintain the ECM within the zG, thus restricting capsular cells from delaminating into the cortex. The latter possibility seems likely as CCDC80 was found to suppress expression of zF-restricted steroidogenic enzymes (Walczak et al., 2014). It is therefore reasonable to hypothesize that CCDC80 within the zG interacts with ECM components that then serve to maintain a structural and molecular environment necessary for propagation of long-term retained progenitor cells. Furthermore, it has been shown that Wnt/β-catenin signaling directly regulates rosette structures in the zG (Leng et al., 2020). Taken together, these data suggest that CCDC80 is an ECM-related Wnt/β-catenin target that plays a key role in maintaining the Wnt-responsive adrenocortical progenitor cell niche. New molecular and

cellular techniques have since been developed, allowing for further research to define this role of CCDC80 in the adrenal cortex.

3.6 Materials and Methods

Single cell RNA sequencing (scRNAseq) Relevant methods pertaining to scRNAseq on mouse adrenocortical Wnt-GFP cells are described in **Chapter 2**.

Immunohistochemistry Adrenals were excised from mice, dissected of surrounding periadrenal fat, and fixed in 4% paraformaldehyde for 2 hours at 4 degrees C. Following PBS washes, adrenals were partially dehydrated stepwise in ethanol and prepared for tissue processing. After processing, tissues were sectioned by microtome into 5 micron sections and dried overnight. For immunohistological staining, adrenal sections were rehydrated stepwise and boiled for 10 minutes in 10 mM citric acid (pH 2.0) and allowed to cool for 20 minutes at room temperature. Sections were then incubated in blocking buffer for 1 hour at room temperature followed by overnight incubation with primary antibodies of interest (summarized in **Table 2.1**). After antibody incubation, antigens were revealed using species-specific DyLight secondary antibodies at a ratio of 1:10,000 in 1X PBS. 4X and 10X images were obtained using an X-Cite Series 120 Q fluorescent lamp on a Nikon Opti-Phot 2 microscope.

Histology Adrenal sections were deparaffinized and dehydrated stepwise followed by brief incubations in Harris' hematoxylin and eosin stains. Brightfield images were obtained using 20X objective on a Nikon Opti-Phot 2 microscope.

Table 3.1. Antibodies used for IHC

Antigen	Species	Blocking buffer	Dilution buffer	Dilution	Source
SF1	Rabbit	PBS + 5% NGS	PBS + 5% NGS	1:1000	Custom made (Proteintech Group)
COUP-TFII	Mouse	M.O.M. blocking reagent (36 uL/1 mL PBS)	M.O.M. protein concentrate (80 uL/1 mL PBS)	1:250	R&D Systems (pp-H7147-00)
CYP11B2	Rabbit	PBS + 5% NGS	PBS + 5% NGS	1:100	Homemade by Celso Gomez-Sanchez
CYP11B1	Mouse	M.O.M. blocking reagent (36 uL/1 mL PBS)	M.O.M. protein concentrate (80 uL/1 mL PBS)	1:200	Homemade by Celso Gomez-Sanchez

Scratch assays Mouse adrenocortical Y1 cells were plated at 200,000cells/well in a 6-well plate and allowed to attach overnight. Cells were transfected with 1 ug of either pkr5 (empty vector) or Ccdc80-HA-myc construct using PEI at a ratio of 3:1. Fresh media was added to cells 6 h later. Using a pipette tip, scratches were made down the center of each well to form a gap between cells. Cells were imaged 24, 48, and 72 hours post-scratch (48, 72, and 96 hours post-transfection) to analyze cell migration over the gap.

Cell transfection and conditioned media experiments HEK293T cells were counted plated on a 6-well plate at a density of 250,000 cells/well and allowed to attach overnight. Cells were then transfected with 1 ug of either pcDNA or Ccdc80-HA-myc construct using PEI at a ratio of 3:1. Protein lysates were harvested 48 h later using RIPA buffer containing protease and phosphatase inhibitors. Cell lysates were incubated on ice for 30

minutes and sonicated briefly halfway through. CM samples were centrifuged to remove cell debris and filtered before use on Western blots.

Heparin sulfate protein precipitation Protocol for precipitation of CCDC80 with heparin agarose beads was adapted from a previous study (Balaj et al., 2015). 1 mg of protein lysate collected 48 hours post-transfection (hpt) and CM samples collected 24 and 48 hpt were incubated overnight with 500 uL of heparin-coated agarose beads. After incubation, beads pelleted by centrifugation (500 x g for 5 min at 4 degrees C) and washed three times with 1X PBS + 0.1% Tween-20. Proteins were unbound from heparin beads by incubating in 4X Laemmli protein sample buffer with freshly added 2-mercaptoethanol for 5 minutes at 95°. Protein samples were collected in supernatants after centrifugation (10,000 x g for 10 minutes at 4°C).

Western blot Protein lysate samples were quantified using Pierce BSA Protein Assay Kit (Thermo, Cat. No. 23227) according to manufacturer’s instructions. Equal protein quantities were boiled for 5 minutes at 95°C in 4X Laemmli sample buffer (Bio-Rad, Cat. No. 161-0747) with freshly added 2-mercaptoethanol. Samples were loaded onto 8% SDS-PAGE gels and transferred to PVDF membranes by standard wet transfer procedures. Membranes were blocked in 1X TBS + 0.1% Tween-20 for 1 hour at room temperature and incubated overnight at 4°C with rocking with the following antibodies: 1:500 anti-HA (CATALOG NUMBER); 1:500 anti-SDC3 (Abcam, ab155952); and 1:5000 anti-β-actin (Thermo-Fisher, A5441). Blots were visualized following incubation with

Odyssey LI-COR IR secondary antibodies (1:10,000) using an Odyssey LI-COR imager and software.

CHAPTER 4 Summary and Future Directions

4.1 Disclosure of relevant publications

Portions of this work are being prepared for publication:

Little III DW*, Borges KS*, Basham KJ, Azova S, O'Connell AE, Dumontet T, LaPensee CR, Breault DT, Hammer GD. WNT2B is essential for adrenal glomerulosa identity and function. In preparation. *co-first author

4.2 Wnt/β-catenin signaling in adrenocortical zonation

Developmental signaling pathways are highly conserved across vertebrate species. Wnt signaling, including canonical (β-catenin-dependent) and non-canonical (Wnt/PCP, Wnt/Ca^{2+}), is activated by morphogenic WNT ligands that are notoriously involved in cell polarity, stem cell proliferation, organogenesis, and differentiation **(section 1.3)**. In addition to its role in development, Wnt/β-catenin signaling remains active throughout adult life to maintain the homeostatic renewal, repair, and function of several tissues, including the adrenal cortex (Kim, A.C. et al., 2008; Berthon et al., 2010). Adrenocortical cells are arranged in centripetal histological and functional zones that produce steroid hormones essential for life **(section 1.4)**. Subcapsular undifferentiated progenitor cells marked by SHH reside in the zona glomerulosa (zG) and subsequently differentiate into *Cyp11b2*-expressing (aldosterone-producing) zG cells, which in turn replenish *Cyp11b1*-expressing (glucocorticoid-producing) cells of the zona fasciculata

(zF). While Wnt/β-catenin signaling is essential for all zG cells, its activity is inhibited as cells transdifferentiate into zF **(Chapter 1)**. Work presented in this dissertation detail the activity of a previously unknown adrenal WNT ligand in both human and mouse that plays a critical role in steroidogenesis and maintenance of the adult adrenal cortex **(Chapter 2)**. Furthermore, we have developed *in vitro* and *in vivo* systems to discover and define the roles of adrenocortical Wnt/β-catenin target genes in progenitor cell maintenance and steroidogenesis **(section 2.10; Chapter 3)**. Here, I will outline the major conclusions of data presented in this thesis in **sections 4.3-4.6** and discuss future directions for this work in **section 4.7**.

4.3 Capsular-cortical unit in zG cell identity and function

β-catenin, the main effector of canonical Wnt signaling, is present in the adrenocortical zG early in development and remains throughout adult life (Berthon et al., 2010; Basham et al., 2019). Previously, *Wnt4* expression was detected in the zG and thought to play the primary role in activating β-catenin. However, loss of *Wnt4* in mice caused a reduced functional capacity of aldosterone-producing zG cells but had no impact on adrenocortical zonation or morphology (Heikilla et al., 2004). Later, the necessity of Wnt/β-catenin potentiator *Rspo3* expressed by the adrenal capsule in zG development (Vidal et al., 2016) supported the capsule as a signaling center that provided key signals for the establishment and maintenance of this essential progenitor cell niche **(section 1.5)**. Indeed, additional studies began showing the presence of *Wnt2b* in the adrenal capsule, leading to the hypothesis that capsular WNT2B activates adrenocortical Wnt/β-catenin activity alongside RSPO3 **(Chapter 2)**.

The loss of *WNT2B* expression in humans is associated with chronic congenital diarrhea and eye abnormalities (O'Connell et al., 2018). In our work, we have uncovered an additional RAAS defect in *WNT2B*-null patients who present with renin-mediated aldosterone compensation **(section 2.4; Table 2.1)**. To define the WNT2B-dependent mechanisms in RAAS and adrenal biology, we generated two mouse models using previously reported *Wnt2b*-floxed mice (Tsukiyama and Yamaguchi 2012). Studies in mice show that developmental loss of *Wnt2b* results in zG depletion, and the homeostatic deletion of *Wnt2b* in the adrenal capsule causes disorganized morphology, dampened Wnt-high activity, and downregulation of essential markers in the zG **(Figures 2.5-2.20)**. Moreover, mice lacking *Wnt2b* have significantly decreased aldosterone-renin ratios and thus provide a relevant model for studying the effects of adrenal *WNT2B* loss in human patients **(sections 2.4-2.9)**. Understanding the implications of WNT2B-activated Wnt/β-catenin signaling in adrenocortical cell biology, particularly as it pertains to individual fates of progenitor and steroid-producing zG cells, is therefore of great importance.

4.4 Identifying novel adrenocortical Wnt/β-catenin target genes

Although the essential role of Wnt/β-catenin signaling in adrenocortical cell biology is undeniable, its scope is far from completely understood. Uncovering Wnt/β-catenin target genes specific to the adrenal cortex will provide greater insight into its roles in progenitor cell maintenance and steroidogenesis, especially as they pertain to human disease. Therefore, in this dissertation work we sought to define the role of a novel β-catenin target gene discovered in our lab **(Chapter 3)** and design protocols utilizing the

newest innovative technologies to distinguish transcriptional programs at the cellular level **(section 2.10)**.

Ccdc80 was discovered as an adrenocortical Wnt/β-catenin target gene and was hypothesized to play a significant role in ECM biology, cell adhesion, and steroidogenesis (Walczak et al., 2014). Early in life, mice lacking Ccdc80 exhibited encapsulated adrenocortical nodules demonstrating typical zonation patterns and steroidogenic capacity **(Figure 3.3)**. However, adult *Ccdc80*-null mice showed neither an increase in the presence of nodules nor disrupted capsular/cortical organization **(Figure 3.4)**. *In vitro* experiments confirmed a CCDC80 interaction with heparin sulfate (HS) chains, but no effect on mouse adrenocortical cell adhesion or migration was observed in the experiments performed **(Figures 3.5-3.7)**. Although no extracellular effect of CCDC80 was established by this work, further study on its role in steroidogenesis is warranted.

To identify new Wnt/β-catenin target genes involved in zG cell biology, we employed single-cell RNA sequencing (scRNAseq) on primary Wnt-responsive cell of the mouse adrenal cortex **(section 2.10)**. Sorted Wnt-GFP cells analyzed by scRNAseq show clearly show the adrenocortical Wnt/β-catenin activity gradient as observed by *Wnt4* expression, which is highest in *Cyp11b2*-concentrated cells and decreases in *Cyp11b1*-high cells of the upper zF **(Figure 2.21-2.23)**. These results align well with our IHC and RNAScope data and capture single cells of the upper zF that are marked by Wnt-moderate activity. Moreover, scRNAseq has provided further insight into zG cell heterogeneity as expression of *Shh*, a marker previously thought to be exclusive to non-steroid-producing progenitor cells, is observed throughout the zG **(Figure 2.24)**. This suggests that while *Shh* transcription is present in all Wnt-responsive zG cells, its

translation may be tightly regulated and occur only in progenitor cells, in which SHH protein expression has previously been shown (King et al., 2009). Finally, RNAseq and gene set enrichment analyses (GSEA) have identified putative markers of proliferative zG cells as well as Wnt/β-catenin target genes that follow the same expression gradient as *Wnt4* **(Figures 2.25-2.27; Table 2.2)**. qPCR results for genes most significantly upregulated in *Cyp11b2*-expressing cells (*Vsnl1*, *Ism1*, and *Ecrg4*) show a downward trend in *Wnt2b* cKO mice compared to Cre-negative *Wnt2b* fl/fl controls, although their potential roles in aldosterone production or cell identity have not yet been explored. Together, our scRNAseq work provides a powerful new way to analyze single zG cell transcriptomes and has allowed us to identify new targets and gene sets of interest that may be pertinent downstream targets in Wnt/β-catenin-associated adrenal diseases.

4.5 Wnt/β-catenin signaling in ACC

Wnt/β-catenin signaling must be tightly regulated in the adrenal cortex. Aberrant activation is associated with tumors of the adrenal cortex, especially adrenocortical carcinoma (ACC), a rare but malignant and often deadly cancer that metastasizes efficiently. Data from The Cancer Genome Atlas (TCGA) on ACC revealed Wnt/β-catenin signaling alterations in 40% of ACCs (Zheng et al., 2016). Mutually exclusive *ZNRF3* deletions and activating *CTNNB1* (β-catenin) mutations represented 20% and 16% of cases, respectively. These mutations are likely dependent upon factors unique to the adrenal cortex as Wnt/β-catenin activity and pathway mutations are largely tissue- and context-specific. Whereas *CTNNB1* mutations and *ZNRF3* deletions make up the majority of Wnt pathway alterations in ACC, deletions of *APC* are rarely observed, although this

alteration affects nearly 70% of CRC cases. The milieu of both paracrine and endocrine factors that regulate β-catenin activity is likely unique to each tissue and therefore may play a fundamental role in the initiation and progression of tumorigenesis. Indeed, unpublished work from our lab suggests that in a tumorigenic context, β-catenin may hijack its usual inhibitors in the adrenal cortex to promote alternative functions necessary for tumor cell growth. Furthermore, it is unknown in which cell population β-catenin mutations initially occur in the adrenal cortex and how these mutations play a role in early adrenocortical tumorigenesis. While the constitutive activity of β-catenin on canonical Wnt signaling has been well established, it is unknown if Wnt-independent functions of β-catenin, such as E-cadherin binding and ECM regulation, are similarly dysregulated to initiate or promote ACC.

Interestingly, while ACCs with high Wnt/β-catenin activity include *ZNRF3*- and *CTNNB1*-altered cases, a large portion lack any alterations associated with Wnt/β-catenin signaling. Therefore, it is possible that WNT ligands play a pathogenic role in activating Wnt/β-catenin signaling in ACC, including *ZNRF3*-deleted tumors. RNA sequencing of ACC cases revealed no significant upregulation of *WNT2B* (Zheng et al., 2016). However, analysis of TCGA and other datasets through the online Gene Expression Profiling Interactive Analysis (GEPIA) shows a significant association between high *WNT2B* expression and disease-free (logrank p=0.0045; HR=2.6) but not overall (logrank p=0.082; HR=2) survival in ACC **(Figure 4.1)**. While it is unknown to what extent WNT2B derived from the adrenal capsule plays a role in adrenocortical tumorigenesis, our studies suggest that it may activate a Wnt/β-catenin signaling cascade by promoting Wnt4 expression to further reinforce β-catenin activity in the cortex. How these ligands are

involved in initiation and/or progression of adrenocortical tumorigenesis in the context of acquired mutations remains unclear. Moreover, a previous study showed the presence of spindle-like cells—perhaps WNT2B-producing cells of the adrenal capsule—invading the hyperplastic adrenal cortex of mice harboring constitutive β-catenin. However, the identity of these cells and their potential for cortical invasion in ACC has not been explored.

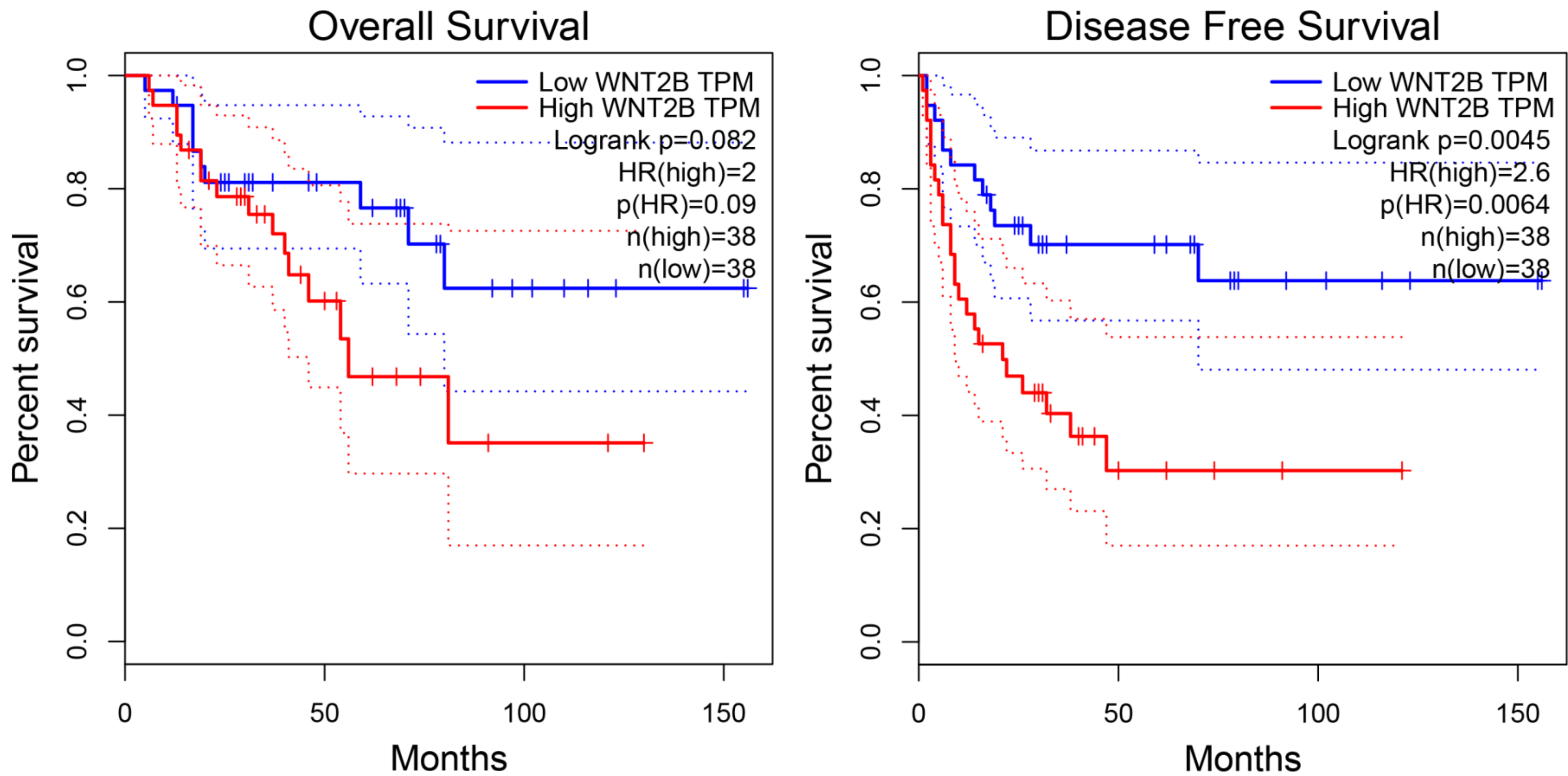


Figure 4.1. High *WNT2B* expression is associated with disease-free survival in ACC
Kaplan-Meier curves depicting overall survival (left) and disease free survival (right) of human ACC patients based on *WNT2B* status. High *WNT2B* expression is significantly associated with disease free survival (logrank p=0.0045; HR=2.6). Data and figure obtained from GEPIA.

4.6 Working model and future directions

Wnt2b expression in the adrenal capsule has been defined and characterized in the bulk of this thesis. WNT2B regulates high Wnt/β-catenin activity in the adrenal zG, a heterogeneous compartment of undifferentiated progenitor cells and aldosterone-producing cells engaged in the physiological RAAS. Given its role as a progenitor cell niche, zG cell identity must be maintained to continually replenish the adult cortex throughout life. Based on our data, our working model suggests capsule-derived WNT2B activates and RSPO3 potentiates Wnt-high signaling in the adrenal zG **(Figure 4.2)**. Downstream intracellular effects of β-catenin lead to maintained regulation of *Shh* and *Cyp11b2* expression. Additionally, β-catenin activates the expression of *Wnt4*, which is suspected to reinforce zG Wnt-high activity. Consequences of dysregulated Wnt/β-catenin activity in the zG include loss of progenitor cell markers and the inability of aldosterone-producing cells to properly engage in the RAAS. However, how *Shh* and *Cyp11b2* are regulated by β-catenin remains unclear. Furthermore, the effects of WNT2B and the consequences of its loss on progenitor and aldosterone-producing cells of the zG requires further study. In the following sections, key experiments to address these and other remaining questions will be outlined.

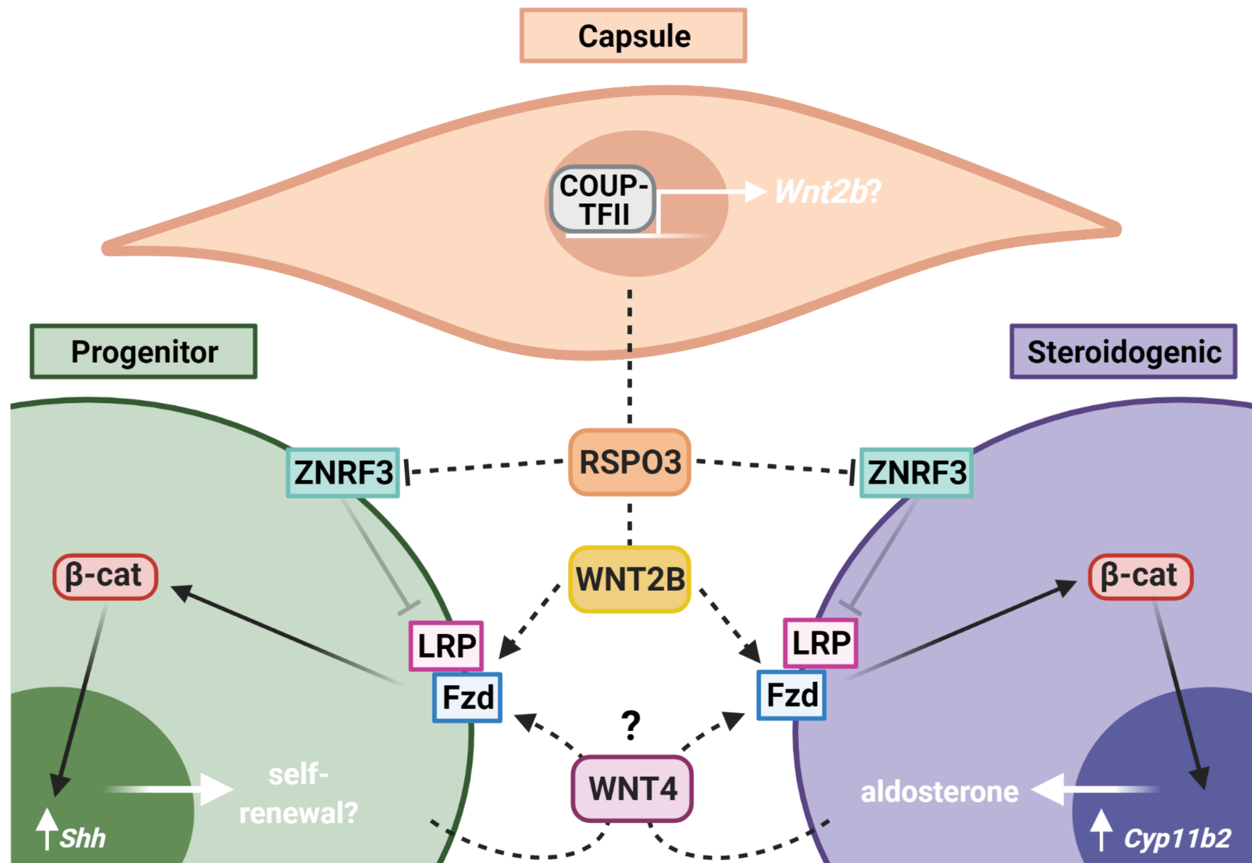


Figure 4.2. Working model: Capsule-derived WNT2B and RSPO3 ligands activate adrenocortical Wnt-high signaling to maintain zG cell-specific identity and function
Capsule cells (orange, top) of the adrenal gland produce and secrete WNT2B and RSPO3. While *Rspo3* transcription is likely regulated by GLI1, the primary activator of *Wnt2b* expression remains elusive, although COUP-TFII is suspected. RSPO3 binds to LGR receptors that cause turnover of ZNRF3, a potent inhibitor of Wnt/β-catenin activation through FZD receptor turnover. WNT2B then binds to transmembrane FZD and LRP receptors, activating cytoplasmic β-catenin and allowing it to translocate to the nucleus. There, β-catenin activates the transcription of Wnt target genes, including *Axin2* and *Lef1*. Our work also demonstrates the β-catenin-dependent activation of both progenitor (left) and steroidogenic (aldosterone-producing; right) cell markers *Shh* and *Cyp11b2*, respectively, although the precise mechanisms are unknown. β-catenin also promotes adrenocortical *Wnt4* expression, which likely acts as reinforcement in the WNT2B-mediated Wnt-high signaling cascade. The specific FZD interactions of both WNT2B and WNT4 in these important zG cell populations remains an exciting area of future research.

4.6.1 Transcriptional regulation of capsular ligands

The necessity of capsule-derived WNT2B and RSPO3 in zG development and maintenance is clear, but their transcriptional regulation in capsular cells has not been explored. Cells of the adrenal capsule express orphan nuclear receptor *Nr2f2* (Coup-TFII), a portion of which also express *Gli1*. While GLI1 is suspected to regulate *Rspo3* transcription, previous unpublished data from our lab suggests *Wnt2b* may be regulated by COUP-TFII (Lewandowski et al., 2015). However, in which cell population(s) each ligand is expressed and in which cells they may be co-expressed has not been explored. First, combined immunofluorescence and RNAScope for COUP-TFII and *Wnt2b*, respectively, would reveal the extent to which they are co-expressed in capsular cells. Then, to test whether COUP-TFII directly activates *Wnt2b* expression, chromatin immunoprecipitation (ChIP) can be performed on adrenal lysates to pull down Coup-TFII and sequence bound DNA portions (Chen et al., 2012). *In vivo* experiments using a small molecule that potently inhibits COUP-TFII can also be used to assess an associated downregulation of *Wnt2b* in the adrenal capsule (Wang et al., 2020). One potential issue with whole adrenal lysates for ChIP experiments is the small proportion of capsule cells relative to the cortex and medulla. To overcome this obstacle, Coup-TFII-expressing adrenal capsule cells can be isolated by FACS from *Nr2f2* (Coup-TFII)-EGFP mice (MMRRC #000289-UNC). While a low number of cells would be isolated from a single mouse, our lab's optimized FACS protocol for scRNAseq would allow for a larger sample size to be obtained and analyzed. GFP-labeled capsule cells from tamoxifen-treated *Gli1-CreERT2*; *mTmG* mice could likewise be sorted and analyzed by scRNAseq. Single-cell RNAseq experiments on Coup-TFII:EGFP cells would also provide deeper insight into the

cell-specific expression patterns and regulators of *Wnt2b* and *Rspo3* in the adrenal capsule.

4.6.2 zG cell-specific Fzd-Wnt interactions

In addition to the presence of *Rspo3* expression alongside *Wnt2b* in the capsule, *Wnt4* expression in the zG necessitates further experimentation to determine WNT ligand-specific effects. Which zG cells respond to WNT2B and/or WNT4 and in which contexts remain unanswered questions. To begin to define the precise interactions of WNT2B and WNT4 in the adrenal zG, single-cell *Fzd* expression must first be determined. Unpublished single molecule ISH data from our lab show the expression patterns of all 10 *Fzds*, but many are lowly expressed throughout the capsule and cortex, thus prohibiting clear single-cell resolution **(see Appendix A)**. Therefore, further utilization of scRNAseq on adrenocortical zG cells, especially GFP-positive cells from *AS* (*Cyp11b2*)-*Cre*; *mTmG* and tamoxifen-treated *Shh-CreERT2*; *mTmG* mouse adrenals can help to assess *Fzd* expression in *Cyp11b2*-positive and -negative cell populations. Parallel immunofluorescent co-staining with CYP11B2 and FZDs of interest would further validate the presence of FZD proteins throughout the zG. Those expressed in CYP11B2-positive and -negative cells could then be pulled down by immunoprecipitation and subjected to proteomic analysis, which would reveal any interactions with WNT2B and/or WNT4. Furthermore, GFP-positive cells isolated from *AS-Cre*; *mTmG* mice can be treated with WNT2B and WNT4 to assess their effects on aldosterone-producing cells *in vitro*. Together, these experiments will further elucidate specific WNT-FZD interactions in the zG to define WNT2B- and WNT4-specific roles.

4.6.3 β-catenin target genes in undifferentiated vs steroid-producing zG cells

Defining target genes of Wnt/β-catenin signaling in the adrenal cortex will not only allow us to understand its scope in zG biology but will also provide potential therapeutic targets in β-catenin-driven adrenal diseases. Analysis of Wnt-GFP adrenocortical cells by scRNAseq provides a powerful foundation for discovering new adrenocortical Wnt/β-catenin target genes and defining their role in zG biology **(section 2.10)**. GSEA has provided further insight into transcriptional programs engaged at the cellular level. Together, these data allow us to determine β-catenin target genes in undifferentiated progenitor cells and differentiated aldosterone-producing cells of the zG. Additional scRNAseq experiments on *Wnt2b* cKO adrenals and subsequent experiments to define the roles of Wnt-high-associated genes in zG cell biology are thus warranted.

4.6.4 Transcriptional regulation of zG markers

SF1 (*Nr5a1*) is the master regulator of steroidogenic gene expression in the adrenal cortex. However, its role, along with the roles of SF1-independent factors, in regulating transcription of zG markers is not completely understood. SF1 has been shown interact with ATF/CREB transcription factors to activate expression of StAR, an enzyme involved in steroid hormone production (Nogueira and Rainey 2010; Martin and Tremblay 2009; Miller 2008). Interestingly, while SF1 activates transcription of *Cyp11b1* in the zF, it is antagonistic to NURR1 (*Nr4a1*)- and NGFIB (*Nr4a2*)-dependent *Cyp11b2* expression in human adrenocortical cells (Bassett et al., 2004). As NURR1 and NGFIB have been shown to interact directly with β-catenin, further studies on their role in *Cyp11b2* transcriptional activation in response to WNT2B and WNT4, the activity of other

transcription factors, and how SF1 regulates this process are needed (Zhang et al., 2016; Yu et al., 2017b).

Even less understood is the transcriptional regulation of *Shh* in the adrenal zG. Our single molecule ISH and scRNAseq studies **(section 2.10)** reveal zG-wide *Shh* transcription, whereas previous data suggest SHH ligands are produced only by a subpopulation of undifferentiated zG progenitor cells (King et al., 2009). Moreover, Shh signaling is activated in mice administered β-catenin-activating agents and is co-activated with Wnt/β-catenin signaling during adrenal regeneration (Finco et al., 2018). Together, these data suggest an adrenal Shh-Wnt signaling relay between the capsule and cortex, but how Wnt/β-catenin signaling activates *Shh* transcription and the molecular mechanisms involved in SHH protein translation in unique zG cells remains unknown. ChIP assays and single-cell ATAC sequencing, a new technology that allows for the analysis of open chromatin regions in unique cell populations, are thus plausible avenues of future research to address Wnt-responsive transcriptional activators of *Cyp11b2* and *Shh* in unique zG cell populations.

4.6.5 Additional role of WNT2B in the gonads

WNT2B is expressed in several mouse tissues in which it plays an important role alongside other WNT ligands (Tsukiyama and Yamaguchi 2012; Goss et al., 2009; Kubo et al., 2003). Considering the importance of WNT2B and WNT4 in the adrenal gland, it is not surprising that studies have also shown a detrimental effect of their loss in the steroidogenic gonads. Indeed, while *WNT4* loss in humans results in sex reversion, kidney, adrenal, and lung dysgenesis (SERKAL) syndrome and androgen excess, one patient with *WNT2B* loss was found to have 46,XX testicular disorder of sex development

(Biason-Lauber et al., 2007; Mandel et al., 2008; O'Connell et al., 2018; Y. J. Zhang et al., 2021b). In line with these studies, steroid hormone analysis in *Wnt2b*-deficient (*Wnt2b* fl/fl) male mice suggest higher testosterone levels at 4-6 months of age **(see Appendix B)**. These data thus necessitate further work to determine the extent of a gonadal phenotype in both male and female *Wnt2b*-deficient mice which act as a relevant model to study the role of WNT2B in proper gonadal development and testosterone production.

4.6.6 Genetic rescue of Wnt2b loss

Through in vitro and in vivo studies outlined in this thesis, we have shown that WNT2B activates β-catenin to maintain zG-restricted Wnt-high activity. To further provide evidence for the canonical nature of WNT2B, a genetic rescue mouse line can be generated and further analyzed. The hypomorphic *Wnt2b*-floxed allele provides us with a unique opportunity to study *Wnt2b* depletion without the addition of a Cre driver. Therefore, $Wnt2b^{fl/fl}$ and *AS-Cre*; $Ctnnb1^{flox(ex3)}$ mice can be crossed to assess the capacity of constitutively active β-catenin to rescue the *Wnt2b* KO phenotype. Constitutively active β-catenin causes zG expansion in mice and leads to adrenocortical hyperplasia and increased tumorigenic capacity (Pignatti et al., 2020; Berthon et al., 2010; Borges et al., 2020). We would expect activated β-catenin to restore Wnt-high signaling activity, cell proliferation, and aldosterone production in the context of *Wnt2b* loss. These experiments would serve to strengthen our results and further illuminate WNT2B activity through β-catenin in the adrenal zG.

4.6.7 Potential effect of WNT2B in ligand-dependent ACC

Given the association of high *WNT2B* expression with decreased disease-free survival **(Figure 4.1)**, further studies are warranted to understand the role of WNT2B and other adrenal WNT ligands in ACC. In addition to further defining its role in activating β-catenin, mouse crosses can also be done to begin to understand the role of WNT2B in WNT ligand-dependent ACC, particularly in the context of *ZNRF3* loss. *Sf1-Cre*; *Znrf3*$^{fl/fl}$ mice exhibit markedly larger adrenals caused by expansion of Wnt-moderate cells of the upper zF (Basham et al., 2019). While activating β-catenin mutations are observed in about 16% of ACCs, *ZNRF3* deletion accounts for 20% of cases, in which loss of this FZD receptor inhibitor may confer higher sensitivity of subcapsular adrenocortical cells to WNT2B and RSPO3 ligands as they act upstream of β-catenin. Therefore, a feasible way to study this potential role of WNT2B in a model of ligand-dependent ACC is to cross *Sf1-Cre*; *Znrf3*$^{fl/fl}$ and *Wnt2b*$^{fl/fl}$ mice. The significant reduction in *Wnt2b* expression is expected to reduce the expansion of Wnt-moderate cells in the context of *Znrf3* loss. However, one caveat to this study is that WNT2B acts primarily on Wnt-high zG cells immediately adjacent to the capsule. Therefore, it is likely that *Wnt4* downregulation in the context of *Wnt2b* reduction would play a significant antagonistic role in Wnt-moderate cell expansion in adrenals lacking *Znrf3*.

APPENDICES

The following appendices include data that do not directly fit elsewhere in this thesis but are pertinent to the work. **Appendix A** includes single molecule ISH data for all Wnt signaling components in the adrenal gland. **Appendix B** outlines hormonal data in Wnt2b-deficient mice and further expands on the potential gonadal phenotype.

APPENDIX A. Expression of Adrenal Wnt Signaling Components

A.1. Rationale, methods, and results

The dependence of the adrenal cortex on Wnt/β-catenin signaling for development, homeostasis, and steroidogenic capacity, as well as its alterations in adrenal diseases, has warranted further evaluation of the expression of Wnt signaling components in the adrenal gland. To better define the scope of both canonical and non-canonical Wnt signaling components in the adrenal capsule and cortex, we utilized single molecule in situ hybridization (smISH, or RNAscope) against all family members of Wnt signaling components: WNTs, FZDs, LRPs, RSPOs, LGRs, destruction complex partners, WNT ligand processing enzymes, and Wnt antagonists **(Figure A.1; work done by K. Basham)**. The following RNAscope analyses were performed using the RNAScope Red Detection Kit (Advanced Bio) on 6-week-old female mouse adrenals. Images were obtained on a brightfield microscope at high magnification. Single transcripts are seen as individual punctate dots. These data have both confirmed previously known adrenal Wnt signaling components and revealed new players that have yet to be explored in adrenal biology.

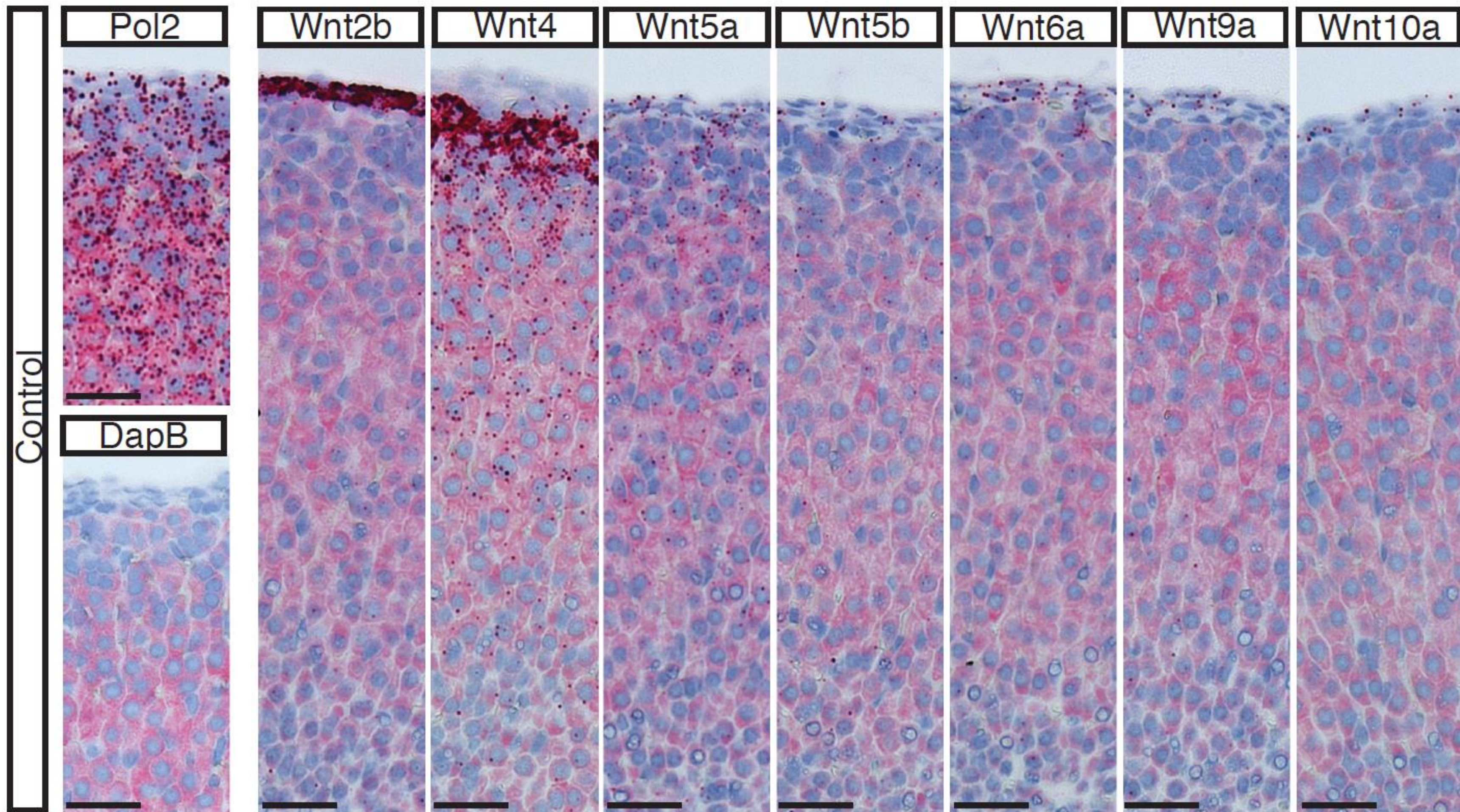
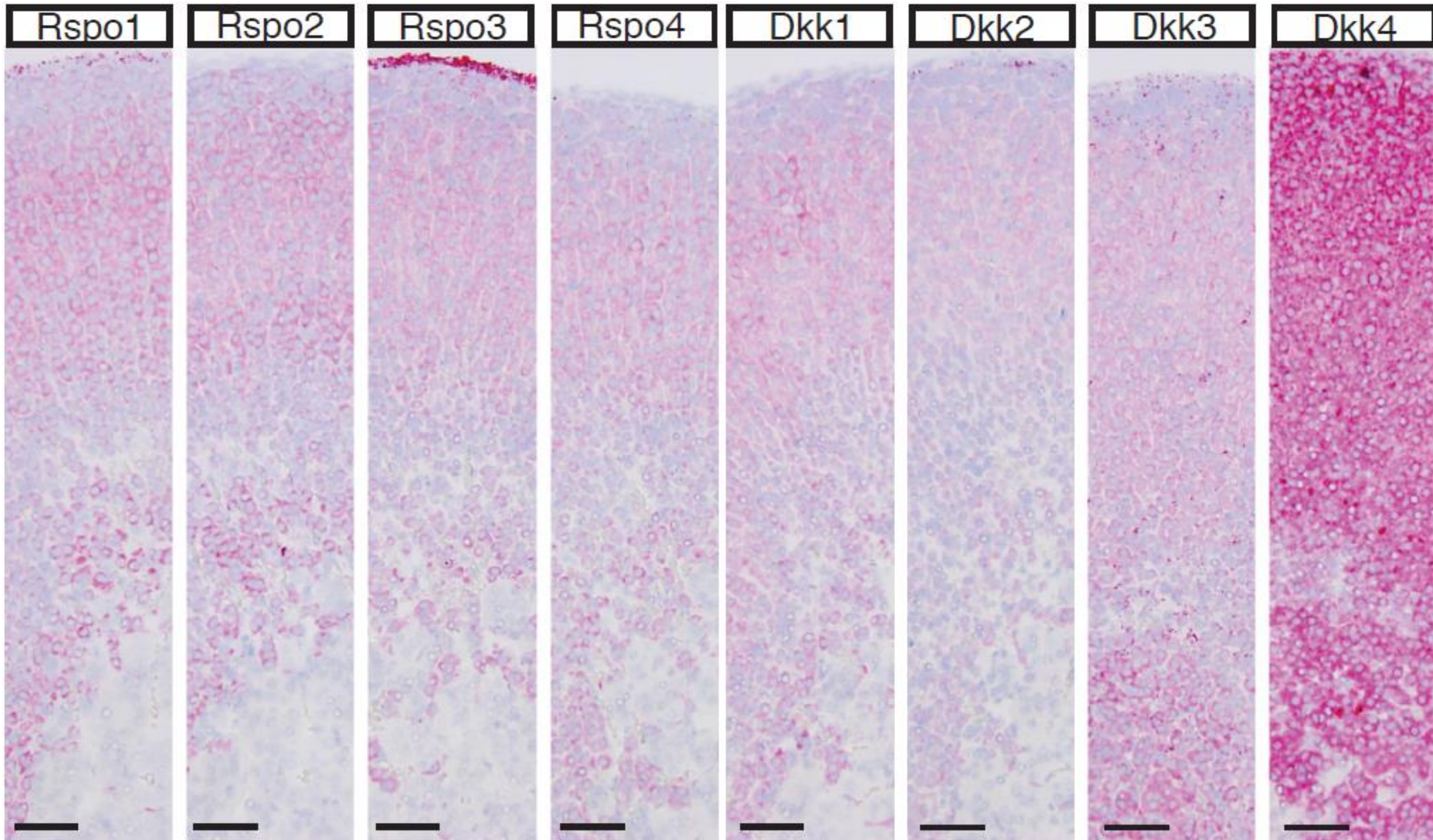


Figure A.1. Single molecule ISH analyses of adrenal Wnt signaling components
RNAscope (smISH) images of Wnt signaling components expressed in the adrenal capsule and cortex. Pol2 serves as a positive control and is expressed in all cells (far left, top). DapB is a bacterial gene used as a negative control (far left, bottom). Panels included on the following pages in order: *Wnt* ligands, *Rspo* ligands, *Fzd*/*Lrp*/*Lgr* receptors, Axins/Wnt processing enzymes, and Wnt antagonists (p.136-141). Work done by K. Basham.



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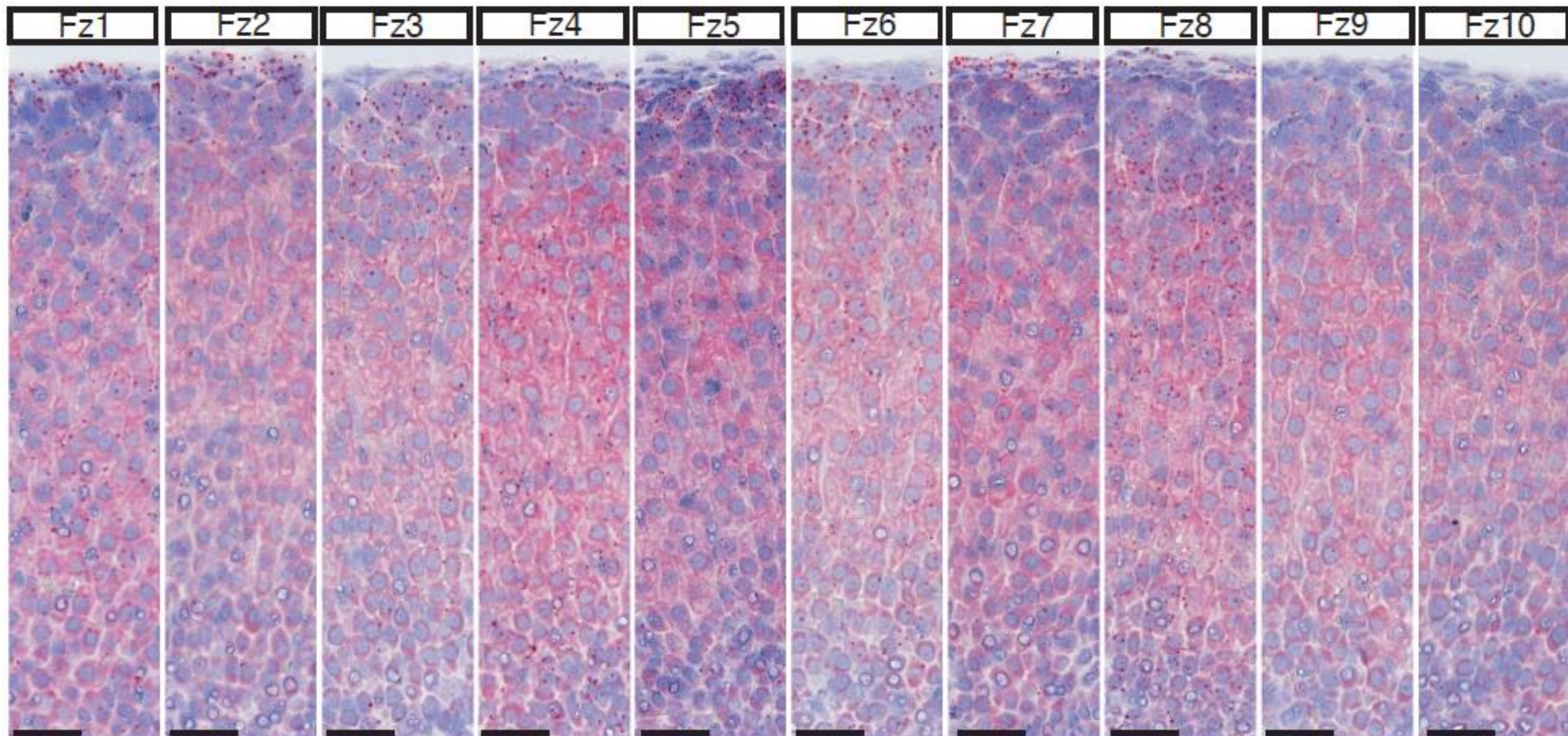

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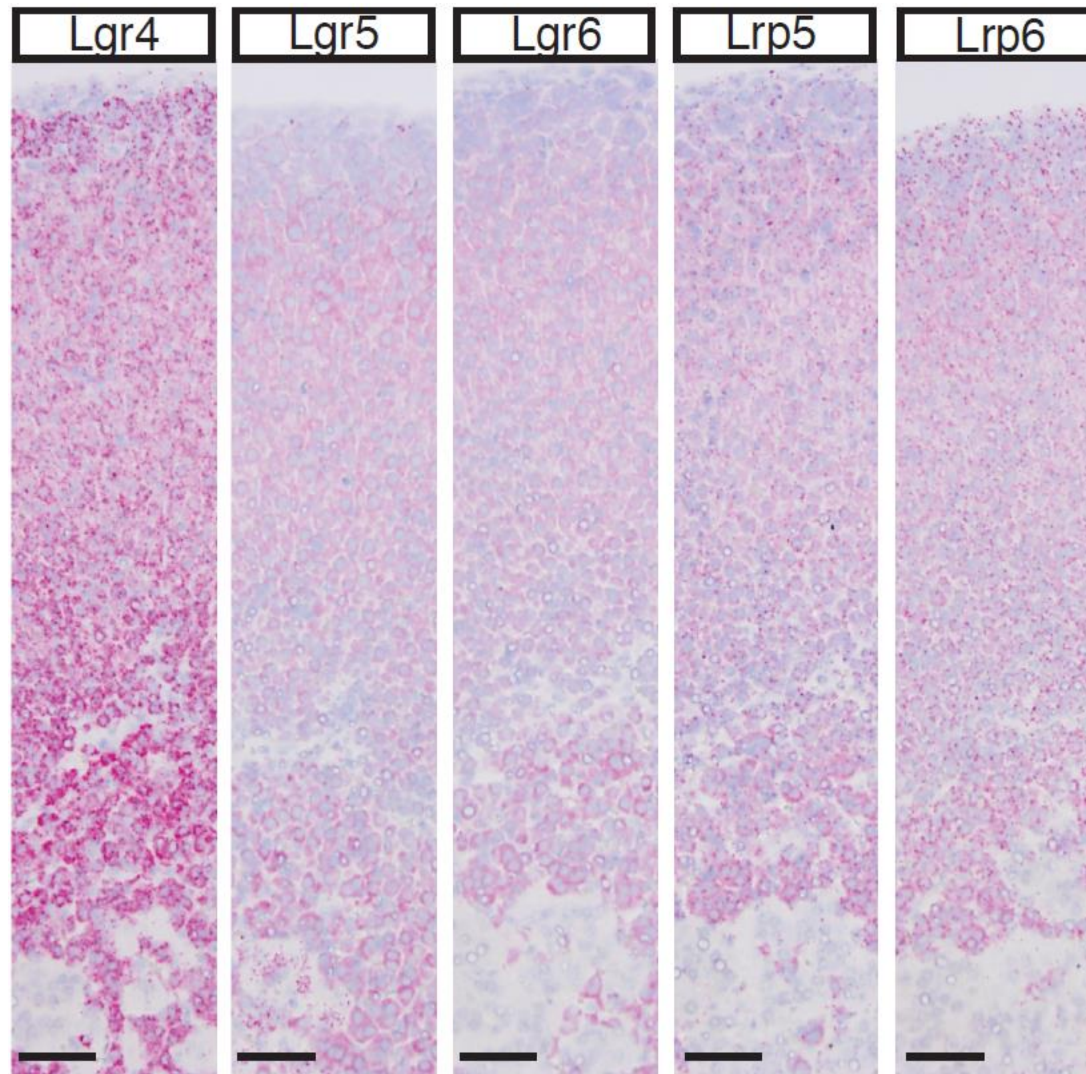

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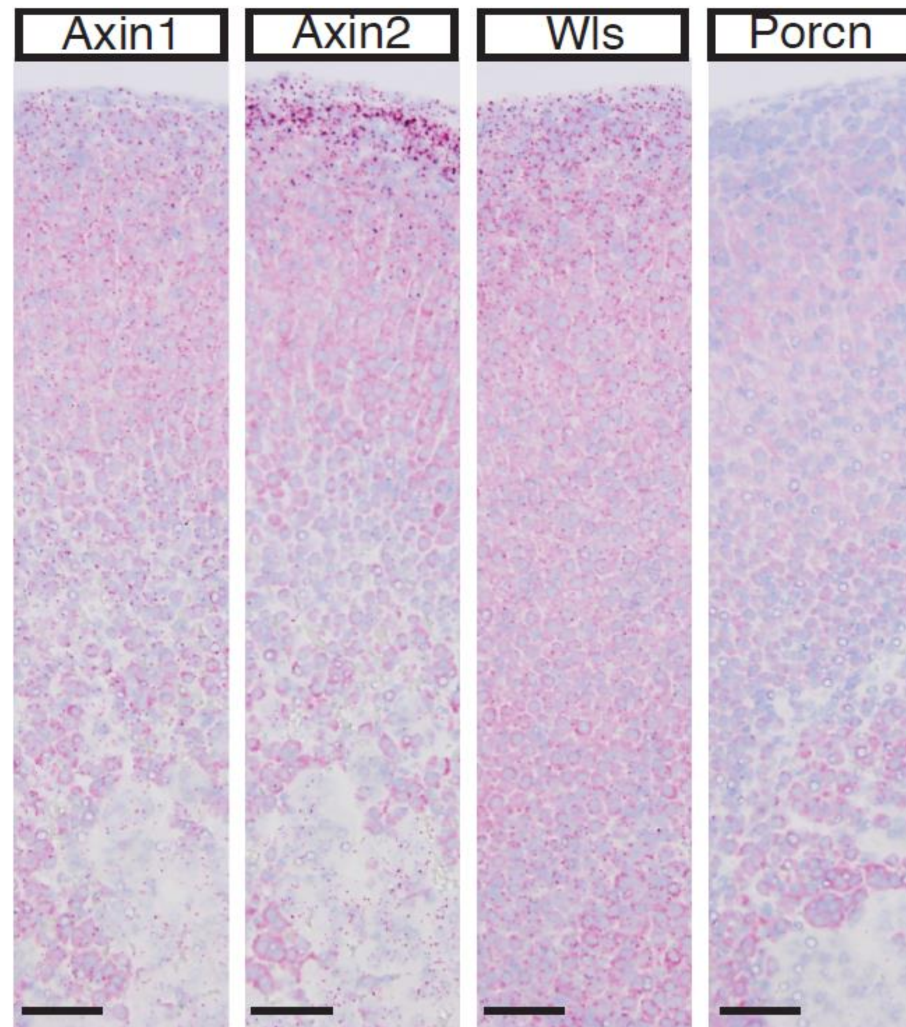

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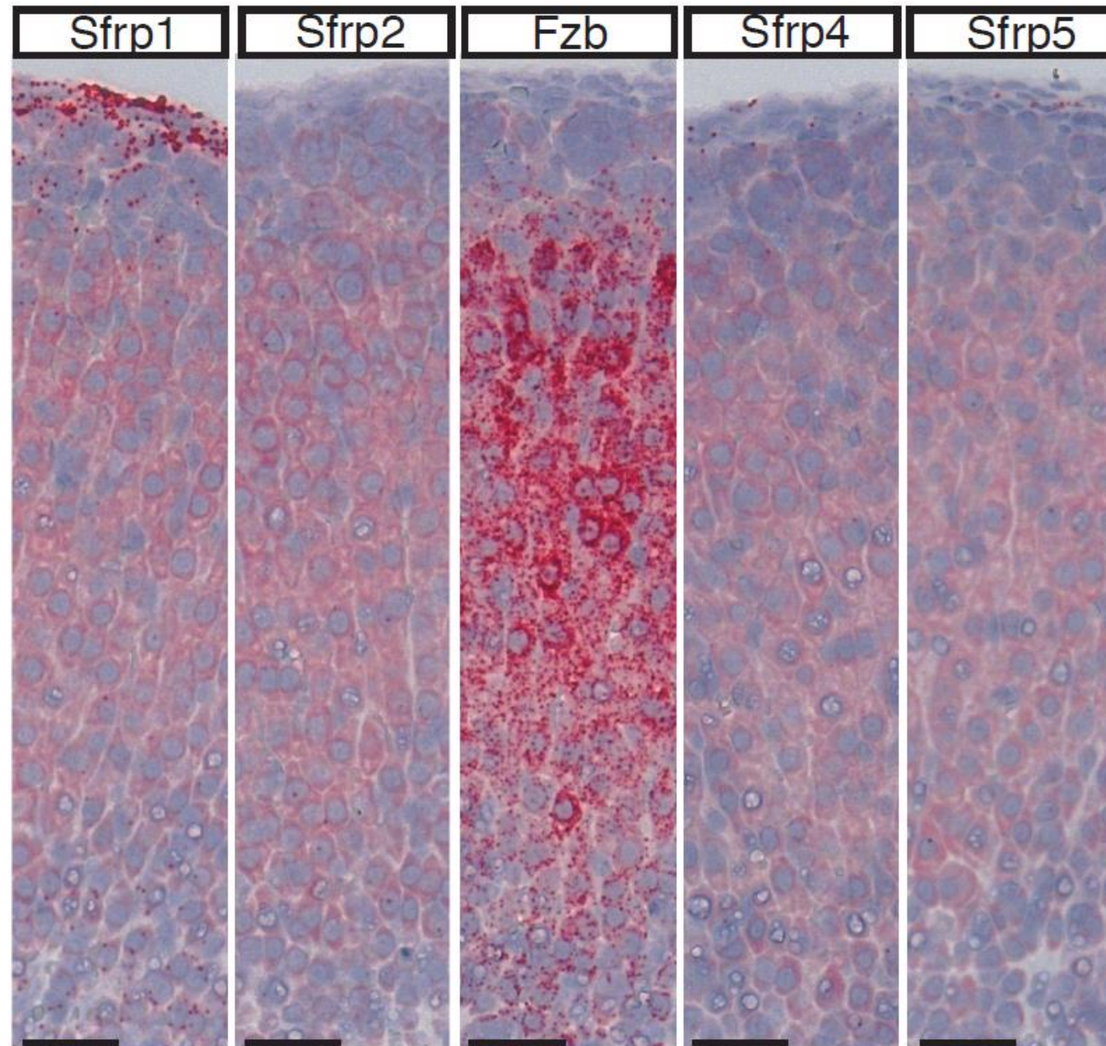

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APPENDIX B. Steroid Hormone Analyses of *Wnt2b*-deficient Mice

B.1. Introduction and rationale

Steroidogenesis is a complex process involving stepwise conversions of cholesterol and its byproducts into steroid and sex hormones. This process requires several enzymes involved in hydroxylation, oxidation, and reduction present in the endoplasmic reticulum and mitochondria of cells in the adrenal cortex, gonads, placenta, and other tissues (reviewed in Miller 2008; Miller and Auchus 2011; and Miller 2017). While the many cholesterol derivatives produced throughout steroidogenesis are beyond the scope of this section, their ultimate conversion into adrenocortical-derived aldosterone and cortisol/corticosterone necessitate their proper production and regulation. Moreover, the decreased capacity of the adrenal zG to produce aldosterone in *Wnt2b*-deficient mice requires further analysis of the prerequisite compounds necessary for its synthesis and that of other steroid hormones. *Wnt2b* expression in mouse ovaries and testis along with the presentation of gonadal defects in some *WNT2B*-null human patients thus warrant further exploration into the role of WNT2B in gonadal steroidogenesis, which will be highlighted in this section (Lin et al., 2001; O'Connell et al., 2018; Y. J. Zhang et al., 2021).

B.2. Methods

Trunk blood from male Wnt2b mice at either 10 weeks (4 week-post tamoxifen treatment) or 4-6 months (untreated) of age was collected as previously described

(Chapter 2). Delta 4 pathway steroid hormones, which include progesterone and its glucocorticoid and mineralocorticoid (11-deoxycorticosterone, corticosterone, 18-hydroxycorticosterone, and aldosterone) and androgen (androstenedione and testosterone) derivatives were then measured by LC/MS-MS **(Figure B.1)**.

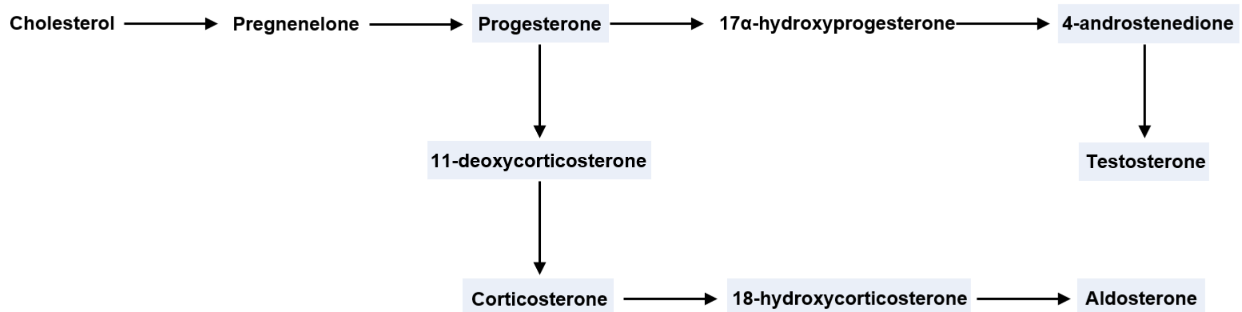

Figure B.1. Steroid hormone synthesis from cholesterol
Simplified schematic showing synthesis of steroid hormones derived from cholesterol. Delta 4 steroid hormones analyzed in this work are highlighted in blue.

B.3. Results and discussion

First, delta 4 steroid hormones of tamoxifen-treated male *Wnt2b* mice (10 w.o.) of various genotypes were compared **(Figure B.2; related to Figure 2.22)**. While no statistical differences are found in any steroid hormones analyzed between genotypes, *Wnt2b*$^{fl/fl}$ and cKO mice show upward trends in progesterone, corticosterone, and testosterone levels compared to both *Gli1-CreERT2*-negative and -positive *Wnt2b*$^{fl/+}$ mice. To determine the long-term effects of *Wnt2b* loss on steroidogenesis, delta 4 steroid hormones were analyzed in WT and *Wnt2b*$^{fl/fl}$ mice at 4-6 months of age. *Wnt2b*$^{fl/fl}$ mice exhibit a similar reduction in *Wnt2b* expression as previously shown **(see Figure 2.8)**. Indeed, *Wnt2b*$^{fl/fl}$ mice have significantly elevated levels of corticosterone, andrestenedione, and testosterone while showing upward trends in progesterone and 11-

deoxycorticosterone **(Figure B.3; related to Figures 2.23-2.24)**. Elevated corticosterone levels in *Wnt2b*$^{fl/fl}$ mice suggest either a zF defect or a chronic stress response due to *Wnt2b* deficiency, warranting further investigation. Additionally, *Wnt2b*$^{fl/fl}$ mice exhibiting elevated testosterone levels suggest a steroidogenic defect in the gonads, providing another avenue to evaluate the effect of *Wnt2b* loss that is relevant to human patients.

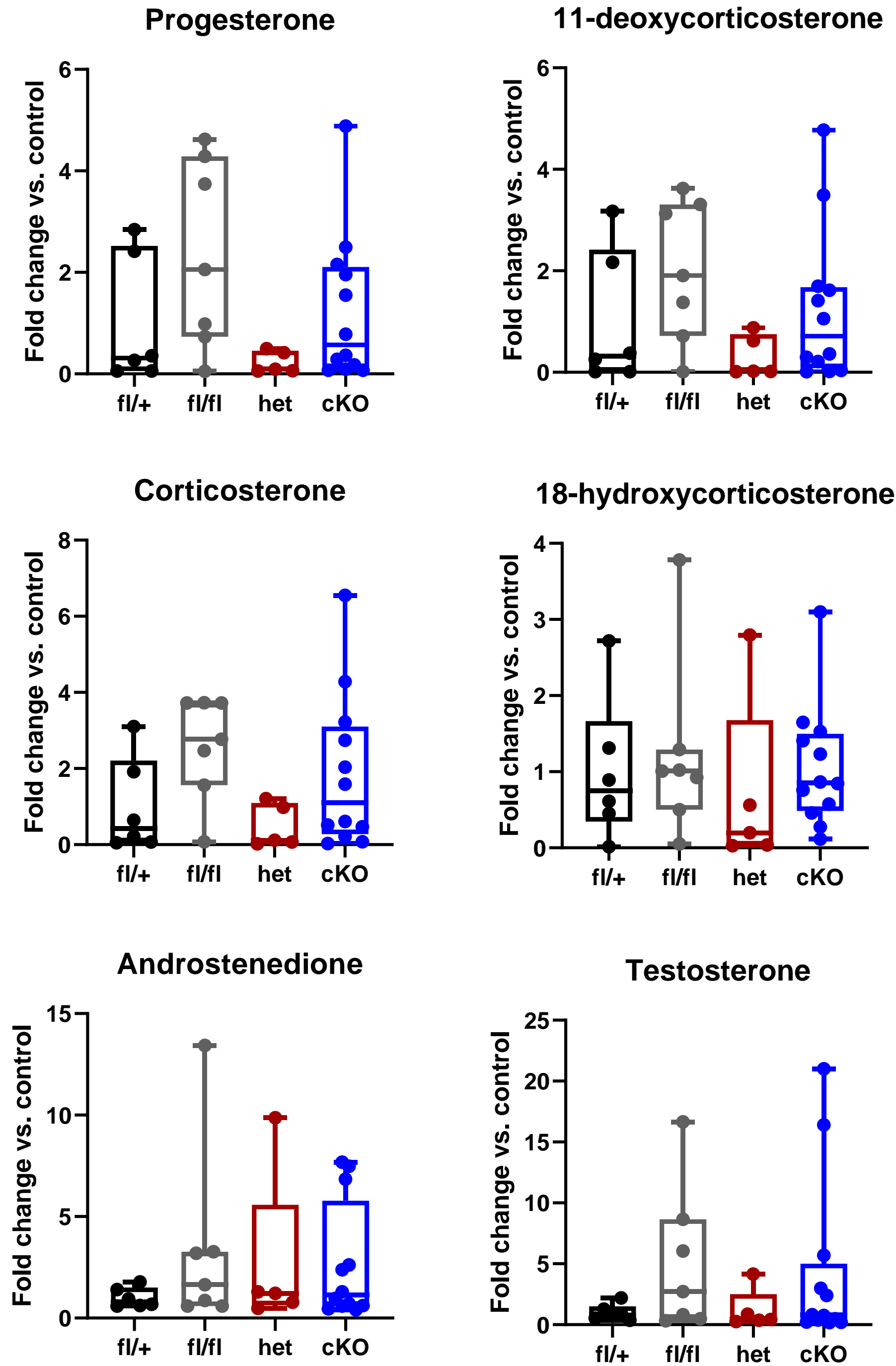

Figure B.2. Delta 4 steroid hormone analysis of *Wnt2b* mice
Fold change of steroid hormones in *Wnt2b*$^{fl/+}$, *Wnt2b*$^{fl/fl}$, heterozygous, and cKO mice analyzed by LC/MS-MS. No significant differences were found in any samples between genotypes, although upward trends in testosterone are observed in *Wnt2b*$^{fl/fl}$ and cKO mice compared to *Wnt2b* heterozygous (+/- Cre) controls. Statistics performed using one-way ANOVA.

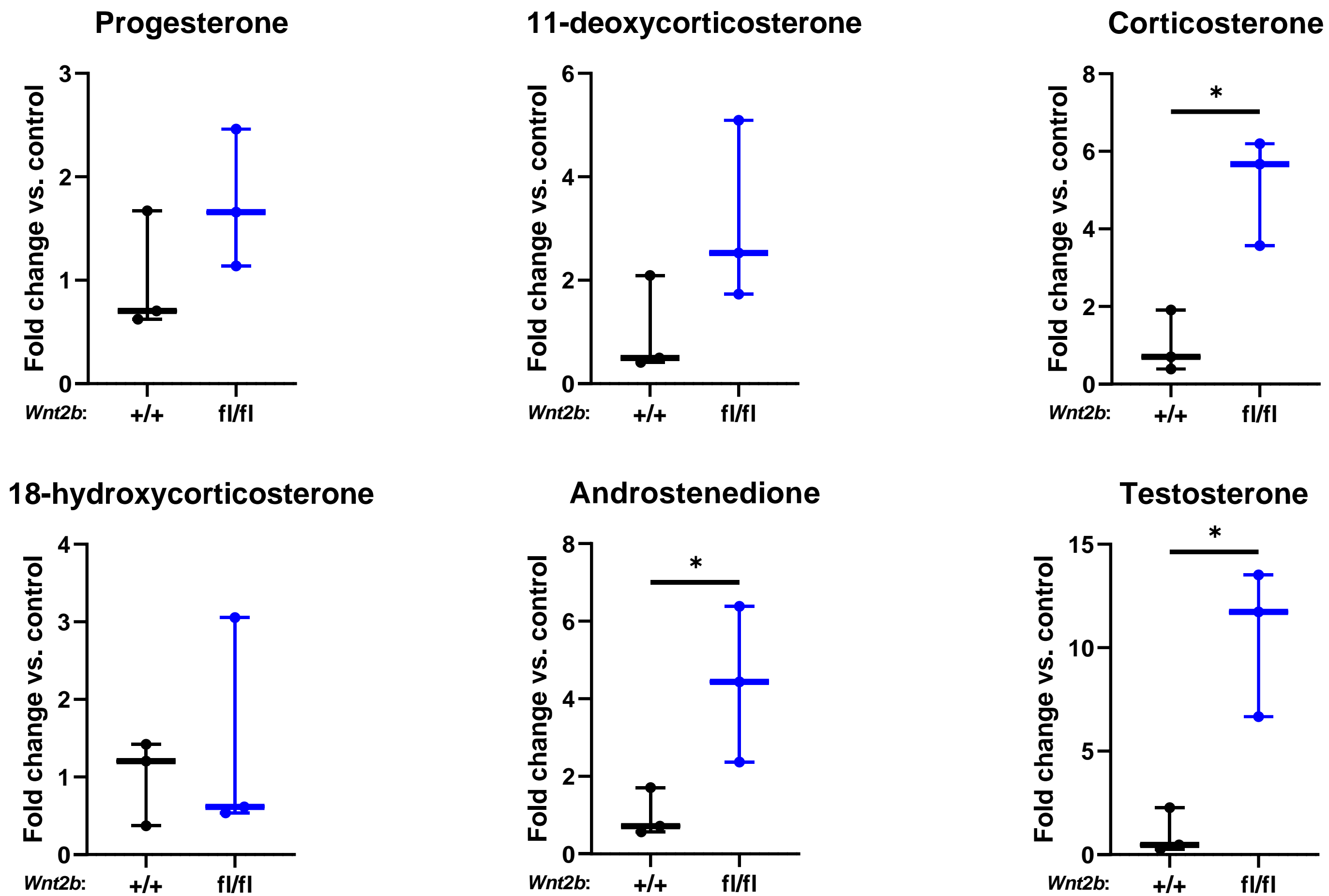

Figure B.3. Steroid hormone levels in aged *Wnt2b*$^{fl/fl}$ mice
Fold change of delta 4 steroid hormones in untreated (Cre-negative) *Wnt2b*$^{fl/fl}$ versus WT mice at 4-6 months of age. *Wnt2b*$^{fl/fl}$ mice have significantly increased levels of corticosterone, andrestenedione, and testosterone, and show upward trends in progesterone and 11-deoxycorticosterone.

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