Contrasting Functions of Notch Signaling In Normal and Pathogenic Adrenal Biology

by

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Abstract

Cell-cell communication is essential for proper development and homeostasis, the dysregulation of which can manifest in developmental defects and/or cancer. The Notch signaling pathway is an evolutionarily conserved pathway that has been implicated in many aspects of these processes. The adrenal glands are endocrine organs that mediate the mammalian stress response and are comprised of the embryological and functionally distinct cortex, which secretes steroid hormones, and medulla, which secretes catecholamine hormones. Benign tumors of the adrenal cortex, adrenocortical adenomas (ACA), are common while adrenocortical carcinomas (ACC) present at much lower frequency but with an extremely poor prognosis. The Notch ligand Jag1 is upregulated in ACCs compared to ACAs and normal adrenals concomitant with upregulations of Notch receptors and target genes. The expression of Jag1 correlates with markers of proliferation and with late stage, aggressive ACC. Upregulated Jag1 mediates a non-cell autonomous effect on cell proliferation through the activation of canonical Notch signaling. In contrast, Notch signaling has no apparent role in the normal adrenal cortex. On the contrary, Notch ligands, receptors, and target genes are expressed in chromaffin cells of the adrenal medulla. Inhibition of canonical Notch signaling in chromaffin cells results in an upregulation of catecholamine-synthesizing enzyme TH while constitutive activation of Notch signaling reduces the expression of the epinehprine-synthesizing enzyme PNMT in chromaffin cells. This thesis interrogates the

contrasting roles of Notch signaling in the molecular biology of the pathogenic adrenal cortex and normal adrenal medulla.

Chapter 1: Introduction*

Introduction to Notch Signaling

A central facet to the regulation of cell biology during development and homeostasis is the receipt and transmission of molecular signals. For example, in adult tissue homeostasis, a common stem/progenitor cell must receive signals to direct it towards a particular cell lineage, instruct it to proliferate and expand or to maintain a quiescent state [1, 2]. In development, it is essential for the appropriate transcriptional program to be activated in order for specification of cell fate. Often times, inappropriate cell signaling results in a disease state such as a developmental syndrome or cancer [1, 2]. Of the myriad signaling pathways, the highly conserved Notch pathway is a crucial player in many of these processes and in a wide range of tissue types.

Notch is unique amongst signaling pathways in several respects (Reviewed in [3-6]). First, the core signaling components are transmembrane proteins that must physically interact and, as a consequence, canonical Notch signaling requires direct cell-cell contact. Second, generation of the active signaling molecule requires a series of proteolytic events culminating in the

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cleavage of the intracellular domain of the Notch receptor itself. Finally, despite a robust activation in numerous systems, there is no amplification of the active signaling molecule. These features allow for an incredible fine-tuning in signaling specificity and as such, the function of Notch in a particular system is highly context-dependent. Nevertheless, much has been learned about the activation and regulation of the core Notch pathway and its function in development, homeostasis, and disease.

Canonical Notch signaling is initiated though receptor-ligand interaction that requires direct contact between adjacent cells and culminates in the release of the active signaling molecule, the cleaved Notch intracellular domain (NICD) (**Fig 1**). Notch receptors are large single-pass type I transmembrane that are highly conserved amongst vertebrates. One receptor gene has been identified in the fly *Drosophila melanogaster*, two in the worm *Caenorhabditis elegans*, and four receptors (Notch1-4) have been identified in mice and humans [4]. Notch receptors are composed of several functional domains but can be grossly divided into two regions: the Notch extracellular domain (NECD) and the Notch transmembrane/intracellular domain (NTMICD) (**Fig 1.1A**). The NECD contains about 29-36 epidermal growth factor (EGF)-like domains that can bind calcium ions and are believed to mediate interaction with Notch ligands [7-9]. Subsequent to the EGF repeats of the NECD is a critical region known as the negative regulatory region (NRR), an unstructured loop composed of three cysteine-rich LIN12-Notch repeats (LNR) and part of the heterodimerization (HD) domain, which may serve to repress Notch receptor activation in the absence of ligands [3,

10]. During its transit through the secretory pathway, the full length Notch receptor is cleaved by Furin-like convertase at the S1 site within the HD domain [11]. The N- and C- terminal regions of the NTMICD and NECD respectively are associated extracellularly though non-covalent interactions. A single transmembrane (TM) domain adjacent to the HD domain of the NTMICD lodges the Notch receptor within the membrane. Following the TM domain and located intracellularly are the RBPjκ association module (RAM), which is required for interaction with the Notch transcriptional machinery, a nuclear localization signal, a seven ankyrin repeats (ANK) domain, a second nuclear localization signal, a transactivation domain (TAD) and a proline/glutamic acid/serine/threonine-rich (PEST) domain, which contains degradation signals responsible for regulation of NICD stability.

Notch ligands are also large single-pass type I transmembrane proteins. While a large number of proteins are emerging as potential Notch ligands, the Delta/Serrate family has been the best characterized in the activation of Notch signaling [12] (**Fig 1.1B**). Within the large extracellular domain of these Notch ligands are a N-terminal Delta/Serrate/Lag-2 (DSL) motif, special EGF repeats known as Delta and OSM-11-like protein (DOS) domain, and tandem EGF-like repeats. The DSL and DOS domains have been shown to be required for binding to Notch receptors [3, 12]. In mice, DSL containing ligands can be further subdivided into two classes that lack a cysteine-rich (CR) domain and include Delta-like (DLL) 1, 3 and 4, or posses a CR domain and include Jagged1/2 (JAG1/2).

Upon ligand-receptor interaction, a series of endocytic and cleavage events occur that eventually results in the release of the NICD from the Notch receptor (**Fig 1.1C**). Ligand-receptor binding results in exposure and cleavage of the S2 site within the HD domain by ADAM metalloproteases. This step is of crucial importance to the regulation of Notch activity but the precise mechanism is unclear. It may involve endocytosis of the ligand/receptor complex, which generates a mechanical force that results in a conformational change of the NRR[13]. S2 cleavage results in an intermediate state containing the remaining membrane-tethered portion of the Notch receptor, called the Notch extracellular truncation (NEXT). Two additional cleavage sites within the TM domain, S3/4, are mediated by γ-secretase, a multiprotein complex composed of presenilin, nicastrin, APH1, and PEN2 [14]. This results in the release of the NICD which translocates to the nucleus and binds to the transcriptional repressor CSL (CBF1/RBPjk in mice, Su(H) in flys, and Lag-1 in worms) [15]. CSL is constitutively bound to Notch target genes and recruits co-repressors in the absence of the NICD. Upon NICD binding to CSL, the essential Notch transcriptional co-activator Mastermind (Mam), or Mastermind-like (Maml) in mice, is recruited to CSL to form a ternary complex which is required for induction of transcription. The most common Notch target genes include the Hairy-enhancer of-split (HES) family of bHLH transcription factors, such as Hes1, Hes5, and Hey1. Interestingly, no signal amplification occurs in Notch signaling and the Notch receptor cannot be used for additional signaling events. Therefore, despite the relative simplicity of the core Notch pathway, the regulation of Notch

activation is complex and involves ligand/receptor localization, post-translational modifications such as glycosylation [16, 17], trafficking and endocytosis [18, 19], and other molecular events [3, 4].

Notch signaling in Development and Disease

The fine regulation of Notch signaling and its high degree of conservation amongst numerous phyla suggest its importance in a variety of developmental processes [6]. Indeed, numerous genetic syndromes in humans have been linked to mutations in Notch alleles (reviewed in [20]). For example, JAG1 mutations have been described as causative of Alagille syndrome, an autosomal-dominant disorder characterized by developmental defects in the liver, heart, skeleton, and face [21, 22]. JAG1 is widely expressed in these tissues during development of human embryos [23, 24]. However, the precise function of Notch signaling in development is highly context dependent and appears to vary from organ to organ. For example, in the intestine, Notch signaling seems to be essential for maintenance of the pluripotency of stems cells located in the crypts of intestinal villi [25, 26]. In contrast, in the hematopoietic system, Notch signaling does not be appeared to be required for the maintenance of pluripotency of hematopoietic stem cells but is required at several steps in T-cell development [27-29]. Furthermore, the importance of Notch signaling in numerous other systems has been described (see reviews: in vasculature [30], in heart [31], in lungs [32], in central nervous system [33]).

Dysregulation of Notch signaling has been implicated in carcinogenesis of a variety of organ systems but as in development, its specific role is context dependent; in some instances acting as a tumor suppressor and other as an oncogene (Reviewed in [6, 34-36]). In contrast to its function in development, the Notch ligand JAG1 has been shown to be upregulated in a variety of cancers such as intestinal tumors [37], hepatocellular carcinoma [38], gastric cancer [39], and colorectal cancer [40, 41]. Furthermore, the upregulation of JAG1 in breast and prostate cancers has been implicated in activation of the cell cycle [42, 43], metastasis [44, 45], and correlated to poor prognosis [45-48]. Similar to the duality of JAG1 in development and disease, activating mutations in the Notch1 receptor has been identified in T-acute lymphoblastic leukemia (T-ALL) and these mutations are believed to be causative in that disease [49, 50]. However, Notch may function as potential tumor suppressor in some systems. Indeed, activation of Notch1 signaling has been shown to induce cell cycle arrest in hepatocellular carcinoma [51], small cell lung cancer [52], and medullary thyroid cancer cells [53]. In the mouse, a tissue-specific loss of Notch1 in the epidermis results in hyperplasia followed by eventual tumor formation suggesting that Notch acts as a tumor suppressor in the skin [54]. Despite its significance in numerous self-renewing organ systems, Notch signaling has never been considered in the adrenal glands.

Basic Biology of the Adrenal Cortex

The coordination of the mammalian stress response is mediated by the adrenal gland, an endocrine organ composed of two embryologically and functionally discrete regions: the adrenal cortex and the adrenal medulla (**Fig 1.2**). The adrenal cortex is of mesodermal lineage and secretes steroid hormones while the adrenal medulla is derived from the neuroectoderm and secretes catecholamines [55]. Since the adrenal cortex and medulla are considered as separate organs, the function, development, and cancers derived from each will be discussed separately.

The outer adrenal cortex is responsible for steroid hormone output. The middle zone of the cortex, the Zona Fasiculata (ZF), is a component of the Hypothalamic-pitiuitary-adrenal (HPA) axis, an endocrine system regulating secretion of the glucocorticoid steroid hormone cortisol (corticosterone in mice) (**Fig 1.2A**) [56]. Coricotropin realeasing factor (CRF) is released from the hypothalamus and acts on corticotropes of the anterior pituitary gland in order to stimulate release of adrenocorticotropic hormone (ACTH), a peptide hormone derived from the processing of the proopiomelanocortin (POMC) peptide. ACTH travels through the blood stream to bind the G-protein coupled receptor melanocortin receptor 2 (MC2R) on the surface of the ZF adrenocortical cell where it induces of a signal transduction cascade that culminates in secretion of cortisol, a steroid hormone involved in glucose utilization and energy homeostasis. Cortisol acts in a negative-feedback loop to regulate the activity of the HPA axis in order to modulate the release of cortisol. Cortisol is secreted

from the ZF, the middle zone of the three functional zones of the adrenal cortex (**Fig 1.2B**). The outer zone, the Zona Glomerulosa (ZG), secretes aldosterone, a steroid hormone involved in sodium retention under regulation of Renin/Angiotensin system. The inner zone, the Zona Reticularis, which is not present in mice, secretes sex steroid precursors such as Dehydroepiandosterone (DHEA) and DHEA-sulfate (DHEA-S).

Development of the Adrenal Cortex

Formation of the adrenal gland occurs in several distinct developmental events and is uniquely dependent on the nuclear receptor NR5a1 (Steroidogenic factor 1, Sf1) [55, 57, 58] (**Fig 1.3**). Indeed, the lack of formation of the adrenal cortex in Sf1 knockout mice underscores the requirement of Sf1 expression for adrenocortical development and function [59]. During the 4^{th} week of gestation in humans (E9.0 in mice), proliferation of mesoderm-derived cells of the coelomic epithelia and underlying mesonephros results in coalescence of the adrenogonadal primordium (AGP), defined by expression of Sf1 [60, 61]. At the 8^{th} week of gestation in humans (E10.5 in mice), the bipotential AGP separates into discrete adrenal primordia (fetal adrenal zone) and gonadal primordial [58, 60]. The segregation of discrete adrenal primordia from the AGP is molecularly defined by a Wilm's tumor 1 (Wt1) and Cited2-mediated upregulation of Sf1 expression [62]. Once separated from the AGP, the adrenal primordia activate Sf1 expression through an entirely different mechanism – the recruitment of the

homeobox protein PKNOX1 (Prep1), homeobox gene 9b (Hox) and pre B-cell leukemia transcription factor 1 (Pbx1) to a fetal adrenal-specific Sf1 enhancer (FAdE) [63]. FAdE-dependent expression of Sf1 in the adrenal primordia is maintained over time through autoregulation by Sf1 itself. Proliferation of the fetal adrenal cortex cells is under the control of fetal pituitary-derived ACTH [64]. However, Insulin-like growth factor 2 (IGF2) is expressed throughout the fetal adrenal cortex and several studies have suggested ACTH mediates some of its effects on proliferation through IGF2 action [65-67].

Concurrent with activation of FAdE-driven Sf1 expression at embryonic day E11.5-12.5 in mice (equivalent to 8-9th week of gestation in humans), neural-crest-derived chromaffin progenitor cells migrate into the central fetal gland and form the adrenal medulla followed by the coalescence of the mesenchymal capsule around fetal adrenal [55, 68]. Before encapsulation is complete, the development of the definitive cortex (definitive zone or adult cortex) is initiated between the capsule and the fetal zone. While the fetal cortex ultimately regresses in all species, the timing of regression is species-specific; in humans the fetal zone regression occurs at birth while in mice the zone persists until puberty in males and the first pregnancy in females [56]. In humans, functional zonation of the adult cortex into unique concentric steroidogenic regions initiates at birth concurrent with the coalescence of the adrenal medulla [69].

Adrenocortical Cancer

Adrenocortical Tumors (ACT) are extremely common neoplasms, the vast majority being benign adrenocortical adenomas (ACA) that occur in as many as 4-7% of the population whereas adrenocortical carcinomas (ACC) are extremely rare (~0.5-2 cases/million) accounting for 0.2% of cancer deaths annually [70-74] (**Fig 1.4**). ACC is typically an aggressive neoplasm with many patients presenting with metastases upon diagnosis [70, 71]. Due to difficulty of early detection and lack of effective treatments for advanced-stage ACC, the prognosis of ACC is tragically unfortunate with the average survival for surgically unresectable tumors at 12-months and the overall 5 year survival is historically less than 10% [75, 76]. The molecular pathogenesis is still unclear but numerous recent insights have revealed several critical factors and signaling pathways.

Together with the characterization of the genetic mutations in family cancer syndromes in which ACC occurs, the expression analyses of sporadic ACC have been extremely informative in identifying common genetic changes within ACC [77]. Beckwith-Wiedemann syndrome (BWS) is a genetic disease that increases susceptibly to a wide range of childhood tumors including ACC, albeit infrequently [78, 79]. BWS has been mapped to numerous genetic alterations of the 11p15 locus, which encodes IGF2 [79-81]. IGF2 is maternally imprinted and thus expression is limited to the paternal allele. Loss of imprinting of the IGF2 locus and resultant upregulation of IGF2 is frequently seen in BWS with similar epigenetic changes observed in sporadic ACC, suggesting a common mechanism responsible for IGF2 upregulation [81-84]. Moreover IGF2

has been consistently identified as the most upregulated gene in both pediatric and sporadic adult ACC [85, 86]. Its cognate receptor Insulin-like Growth Factor receptor 1 (IGFR1) is also frequently upregulated whereas the related ligand Insulin-like Growth Factor 1 (IGF1) is not [87, 88]. Indeed, IGFR1 is a potential therapeutic target as pharmacologic inhibition of the IGFR1 results in inhibition of adrenocortical tumor cell growth both in culture and in xenograft model [89]. Moreover, Zac1, an additional epigenetically regulated gene involved in regulation of an IGF2 network of imprinted genes that participate in stem cell maintenance is one of the most downregulated genes in pediatric ACC [90, 91]. Together these data support a role of the IGF2 signaling pathway in adrenocortical carcinoma initiation and/or maintenance.

The Wnt/β-Catenin pathway has emerged in recent years to be a major regulator of both adrenocortical homeostasis and tumorigenesis. Wnt signaling is initiated through binding of Wnt ligands to cognate Frizzled (Fzd) receptors which results in inactivation of GSK-3β/APC/Axin degradation complex and subsequent stabilization of the active signaling molecule, β-Catenin, which translocates to the nucleus, bind to TCF/LEF transcription factors to ultimately initiate transcription of Wnt-responsive target genes [92]. β-catenin is expressed exclusively in the peripheral adrenal cortex of the developing and adult adrenal gland [93]. The slow disappearance of the adrenal cortex in adrenal-specific β-catenin null mice, together with the restricted subcapsular activation of β-catenin suggests a role of Wnt ligands in maintaining adrenocortical homeostasis through regulation of peripheral stem/progenitor cells [93].

Dysregulation of Wnt/β-catenin signaling has also been implicated as an initiating event in adrenocortical tumorigenesis. Adenomatis polyposis coli (APC), a critical component of the β-catenin destruction complex, is the causative mutation in familial adenomatous polyposis coli, a colon cancer syndrome that frequently manifests with ACTs [94, 95]. Loss of APC has been shown to result in stabilization and constitutive activation of β-catenin and thus could be a possible mechanism for the nuclear accumulation of β-catenin seen in ACA and ACC [96]. Because APC acts as a tumor suppressor and requires inactivation of both alleles, it is not unexpected that APC mutations are rare in sporadic ACC [96, 97].

Indeed it is not APC but β-catenin that is commonly dysregulated in sporadic ACTs, both benign ACAs and malignant ACCs. 15-25% of ACA and ACC have been shown to exhibit stabilized nuclear β-catenin [98]. While a majority of these ACTs with nuclear β-catenin have been found to harbor activating mutations on β-catenin, a subset do not, suggesting perturbation of additional upstream mechanisms of Wnt activation (beyond APC and β-catenin mutations) are involved in the constitutive stabilization of β-catenin [99-101]. For example, in the highly inbred mouse strain DBA2/J, in which gonadectomy has been shown to induce subcapsular hyperplasia followed by *bona fide* adrenocortical tumorigenesis, a loss of the Wnt antagonist and putative tumor suppressor SFRP1 (Secreted Frizzled-Related Protein 1) was observed in the post-gonadectomy ACTs suggesting a dysregulation of Wnt signaling [102].

Moreover, in a recent screen of a large cohort of human cancers from a variety tissue types the downregulation of SFRP1 was observed[103]. Unfortunately, ACTs were not included in this cohort and retrospective review of previous ACC cDNA microarray studies does not support SFRP1 as a significantly downregulated gene in sporadic ACC with the caveat that SFRP1 expression has not been specifically analyzed in the cohort of ACC exhibiting abnormal β-catenin nuclear accumulation [86].

The lack of β-catenin mutations in the majority of ACAs (75-85%) suggests that there are indeed other mechanisms of ACT initiation and progression. Moreover, the presence of β-catenin mutations in both benign ACA and malignant ACC suggests that Wnt/β-catenin dysregulation is not sufficient for ACC formation. Indeed, the forced constitutive expression of activated β-catenin in the mouse adrenal cortex results in adrenal hyperplasia and benign tumors but not ACC in aged animals [104]. Together these data support the hypothesis that β-catenin serves as an early genetic abnormality that initiates hyperplastic growth of adrenocortical cells but is not sufficient to drive development of malignant neoplasm but may allow for the accumulation of additional genetic hits such as upregulation of IGF2. To test this hypothesis, our group recently published a study in which β-catenin was stabilized in adrenocortical cells, through ablation of the APC gene, concurrent with an upregulation in IGF2, through a loss of imprinting of the IGF2 locus [105]. In mice with stabilized β-catenin, we identified adrenal hyperplasia and microscopic and macroscopic adenomas but in combination with upregulated IGF2, an increase in macroscopic adenomas and

one carcinoma were observed. These data show that β-catenin stabilization is sufficient to drive adrenocortical hyperplasia and, in the presence of upregulated IGF2, develop adrenocortical tumors. However, the lack of complete penetrance of *bona fide* ACC suggests the involvement of other genes and signaling pathways in adrenocortical tumorigenesis. **Chapter 2** of this thesis interrogates the role of the Notch signaling pathway in ACC.

Basic Biology of the Adrenal Medulla

The adrenal medulla is of distinct ontogeny and function from the adrenal cortex. While the cortex is mesoderm derived and composed of steroidogenic cells, the adrenal medulla is of the neuroectodermal lineage and is composed of modified sympathetic neurons called chromaffin cells [106]. The adrenal medulla is best characterized as mediating the "fight or flight" response through release of the catecholamine hormones epinephrine (adrenaline) and norepinephrine (noradrenaline) [107] (**Fig 1.6A**). The neuroendocrine chromaffin cells of the adrenal medulla and extra-adrenal paraganglia, such as the suprarenal ganglia and the "organ of Zuckerkandl", possess the capability to synthesize, store, release, and re-uptake catecholamines. Catecholamines are biosynthesized in a multi-enzyme process that has been well characterized (See reviews [107, 108]) (**Fig 1.5**). In brief, the rate-limiting enzyme tyrosine hydroxylase (TH), which is expressed in every cell of the adrenal medulla and a marker of sympathetic neurons in general [109-112], converts the amino acid L-tyrosine into L-dopa,

which is in turn converted into dopamine, by aromatic amino acid decarboxylase (DOPA decarboxylase, AADC). Dopamine is metabolized to norepinephrine by Dopamine β -hydroxylase (DBH), which is then converted to epinephrine by phenylethanolamine N-Methyl transferase (PNMT). Of significance, PNMT expression is largely restricted to the adrenal medulla and its presence or absence defines subpopulations of chromaffin cells that either primarily secretes epinephrine (adrenergic) or norepinephrine (noradrenergic) respectively [109, 110] (**Fig 1.6B**). The main catecholamine hormone secreted from the adrenal medulla is epinephrine (i.e. the medulla is predominantly adrenergic) but the relative percentage of chromaffin cells that are adrenergic or noradrenergic appears to vary from species to species [113]. Catecholamines are packaged into secretory granules that are released into the blood stream through exocytosis [107]. Vesicular monoamine transporters (VMATs), a family of transporters specific to epinephrine, norepinephrine, and dopamine, are required for transport of catecholamines into granules. Two isoform of VMATs exists (VMAT1/2) but VMAT1 has been posited as the main isoform expressed in chromaffin cells [114].

CAs are often packaged and co-released with several different peptide hormones (**Fig 1.6B**). Chromogranins (Chr) are a family of small peptide hormones enriched in acidic amino acids that exist in two main isoforms (ChrA and ChrB) [110, 115]. Both ChrA and ChrB appear to be expressed exclusively in secretory granules, with ChrA immunohistochemically as the most robustly expressed in human chromaffin cells (ChrB in mice) and located primarily in

adrenergic versus noradrenergic cells [115-117]. ChrA/B have been shown to mediate a wide range of cardiovascular effects [115]. Neuropeptide Y (NPY) is another peptide hormone concomitantly secreted with epinephrine and norepinephrine, and in addition to peripheral effects, may feedback through its cognate receptor NPY Y_1 on chromaffin cells to modulate catecholamine release [118, 119].

The thoracic splanchnic nerve, of the sympathetic division of the autonomic nervous systems, which releases the neurotransmitter acetylcholine (ACh), directly innervates the adrenal medulla and is responsible for the acute stimulation of catecholamine release [120, 121] (**Fig 1.6A**). ACh binds to nicotinic ACh receptors (NAchR) expressed on chromaffin cells and induces depolarization of chromaffin cells and, in a mechanism similar to neurotransmitter stimulation of neurons, culminates if exocytosis of chromaffin granules [107]. In addition to ACh, the splanchnic-adrenal synapse releases peptide hormones such as pituitary adenylate cyclase-activating peptide (PACAP), which regulates a variety of processes in chromaffin cells through its cognate receptor PAC1 [122, 123]. The significance of PACAP stimulation of chromaffin cells is only recently beginning to be appreciated [123].

Inactivation of catecholamines following secretion occurs through several mechanisms. A series of enzymes are able to metabolically convert catecholamines into inactive intermediates, which can then be excreted through the urine. Monoamine oxidases (MaOs) exists in two isoforms (MaoA/B) which are largely active in peripheral sympathetic systems and are involved in

inactivation of epinephrine to and norepinephrine to 3,4-dihydroxphenyl glycoaldehyde (DHPG) which is subsequently converted to vanillymanedlic acid (VMA) and 3-methoxy-4-hydroxyphenylglycol (MHPG), which are excreted in the urine [107]. Catechol-O-methyl transferase (COMT) appears to be the predominant catecholamine-inactivating enzyme in chromaffin cells and converts epinephrine and norepinephrine into the excretable metabolites metanephrine (ME) and normetanephrine (NME) respectively [124, 125]. However, the metabolism of to ME/NME represents a long-term strategy for inactivation of circulating catecholamines. A more immediate response is the sequestration of catecholamines back into chromaffin cells through plasma membrane transporters of the solute carrier (SLC) family such as the noradrenaline transporter 1 (norepinephrine transporter, SLC6A2, NAT1 or NET1) and dopamine transporter (SLC6A3, DAT1) (**Fig 1.6B**). Both NAT1 and DAT1 are expressed throughout the adrenal medulla and are selective to both epinephrine and norepinephrine [126]. Interestingly, NAT1 expression seems to colocalize with PNMT-expressing chromaffin cells, suggesting a specific function in adrenergic chromaffin cells [126, 127].

Development of Chromaffin Cells from the Neural Crest

Chromaffin cells are derived from the neural crest (NC), a population of transient progenitor cells of neuroectodermal lineage [128, 129] (**Fig 1.7**). The NC cells are pluripotent progenitor cells derived from the junction of the neural

tube and the dorsal ectoderm and give rise to numerous cell types in addition to chromaffin cells including neurons and glia of the enteric and sympathetic division of the autonomic nervous system, melanocytes, skeletal and cartaliginous components of the face, C-type thyroid cells, and others (See reviews, [106, 130-132]). The NC is specified as early as post-gastrulation [133, 134]. In a poorly understood process, NC cells delaminate from the dorsal side of the neural tube following neurulation. One NC subpopulation, sympathoadrenal (SA) progenitors, including progenitors of the sympathetic nervous system, extra-adrenal paraganglia, and chromaffin cells, migrate towards the dorsal aorta (DO) concurrent with expression of the transcription factor aschaete-scute homolog 1 (Ash1, Mash1 in mice) [135-137] (**Fig 1.7A, B**). Bone morphogenetic proteins (BMPs), specifically BMP-2, 4 and 7, released from the DO have been best characterized in specification of SA lineage in the chick embryo, possibly through induction and/or maintenance of Mash1 expression [138-141]. Interestingly, BMPs have also been implicated in initiation in NC migration [142].

In addition to Mash1, a host of other transcription factors has been implicated in specification of the SA lineage (**Fig 1.7B**). The expression of the homedomain transcription factor Phox2B has been identified in all peripheral sympathetic neurons and appears to be required for their development [143, 144]. In a process that appears to be independent of Phox2B expression, Mash1 induces the expression of Phox2A, which is required for TH and DBH expression, markers of sympathetic neurons and chromaffin cells [140, 145-147]. Gata

transcription factors, dHand, and others have also been implicated in chromaffin cell differentiation post-Mash1/Phox2B/Phox2A induction [148-150].

However, the spatiotemporal initiation of Mash1 and Phox2B in SA progenitors and how they contribute to chromaffin cell-specific differentiation program is still unclear. In some SA progenitors, it appears that Phox2B expression may precede Mash1 expression [147, 151]. Mash1 expression is still detectable after migration of SA progenitors into the adrenal primordia in contrast to loss of Mash1 observed in differentiated sympathetic neurons [140, 152, 153]. In Mash1 deficient mice, by E13.5, most cells of the medulla lack TH expression but most of the remaining TH-positive cells express Phox2B [152]. Furthermore, despite the loss of TH expression, some chromaffin progenitors still express Phox2A and dHand, two transcription factors expressed after Mash1 induction, while the neuronal marker c-Ret is not downregulated (i.e. the downregulation of c-Ret is a common molecular change in chromaffin cell maturation) [152]. These data suggests a majority of chromaffin progenitors require Mash1 for suppression of the sympathetic phenotype and subsequent differentiation to mature chromaffin cells but a distinct subpopulation may only require Phox2B. In contrast, in Phox2B deficient mice, chromaffin progenitors appear to migrate to the adrenal primordia by E12.5 but reduce in number and become very sparse by E16.5, concurrent with an increase in apoptosis [151]. At E12.5, Mash1 is still detectable in Phox2B deficient cells but by E14.5, Mash1, Phox2A, dHand, and TH expression are completely absent in virtually every chromaffin cell [151]. These data suggest Phox2B may precede Mash1 in chromaffin cell differentiation

and is the predominant transcription factor in maintenance of chromaffin cell fate [151]. However, these data conflict with the previous study that identified a Mash1-dependent chromaffin population. Nevertheless, the specific transcriptional program that defines sympathetic neurons versus chromaffin cells is still unclear and remains to be elucidated (see reviews [106, 130]) but the preceding studies suggest that multiple progenitors may give rise to mature chromaffin cells.

The Role of the Adrenal Cortex in Development of the Adrenal Medulla

The role of the cortical-chromaffin cell interactions in medullary has been the subject of much debate [154]. It was postulated that glucocorticoids secreted from the fetal adrenal cortex were required for the maturation of chromaffin cells through the induction of PNMT expression, as was shown in isolated rat adrenal chromaffin cell progenitors [155, 156]. These data were validated *in vivo* with the generation of mouse line with targeted disruption of Exon2 of the glucocorticoid receptor (GR) [157]. The medulla in GR-deficient mice was reduced in size and lacked functional PNMT-expressing adrenergic chromaffin cells [157]. However, since this mouse was believed to incompletely ablate GR expression, a second study reported a mouse line in which Exons 3 was deleted [158]. In these mice, the numbers of chromaffin progenitors, as well as expression of molecular hallmarks specific to developing chromaffin cells, appear to be unaltered, which argues against the role of glucocorticoids in medullary development. However,

GR-deficient mice fail to express PNMT that suggests that cortisol is required for complete maturation of chromaffin cells [158]. These data were confirmed in a conditional knockout mouse of GR in DBH-positive cells, which lacked PNMT-positive chromaffin cells [159]. Conversely, TH-null mice, supplemented with L-DOPA during development to bypass the embryonic lethality of these mice, exhibited reduced plasma corticosterone levels suggesting reciprocal feedback from medulla to cortex [111].

Despite these findings, the role of cortical-chromaffin cell interactions in medullary development is still ambiguous. In Sf1 knockout or Sf1 heterozygote mice, chromaffin cells still migrate to the area where the adrenal gland forms, despite the absence of mature Sf1-positive cortical cells [160, 161]. Furthermore, these chromaffin still express molecular markers such as TH but, consistent with the previous studies, lack PNMT expression. Additionally, downregulation of SCG10, a marker of sympathetic neuronal development, was still observed but downregulation of c-Ret was not [160, 161]. These studies confirm that corticosterone is required for PNMT expression. In a recent study examining the status of the adrenal medulla in a variety of mouse models in which cortical development is disrupted or ablated, chromaffin cells migrated and differentiated normally in many of the mouse crosses analyzed [162]. Taken together, these data suggest that development of the adrenal medulla appears to be independent of the cortex, with the exception of induction of expression of PNMT. Regulation of catecholamine and/or glucocorticoid biosynthesis may involve cross talk between the cortex and medulla [109, 154].

Notch Signaling in Tumors of the Adrenal Medulla

Pheochromocytomas (PCC) are chromaffin cell neoplasms that secrete catecholamines [163, 164]. PCC are rare (2-8 cases/million/year), but generally benign tumors, that most commonly manifest in the adrenal medulla (~90% of cases) and less frequently in extra-adrenal ganglia (paragangliomas, PGL) [163, 164]. PCC have classically been shown to clinically manifest with hypertension, headaches, palpitations, and diaphoresis as a consequence of hypersecretion of catecholamines but, due to a differential catecholamine profile for each tumor, symptomatic presentation is actually quite variable [165-168]. The heterogeneity in catecholamine production is a consequence of the appropriate expression of catecholamine biosynthetic enzymes (i.e. epinephrine-producing PCC express PNMT while norepinephrine-producing PCC do not) [169, 170]. Most PCC are benign, well encapsulated tumors though about 25% present with metastases, but the morbidity and mortality is high in PCC due to symptoms associated with catecholamine secretion such as hypertension, stroke and myocardial infarction [163, 164].

While most PCC are sporadic, about 25-30% of PCC occur in hereditary cancer syndromes [171]. Several syndromes have been identified, but only two will be discussed here. von Hippel-Lindau (vHL) syndrome is autosomal dominant cancer susceptibility syndrome that presents with an incidence of about 1 in 36,000 birth/year and is caused by inactivating mutations in the *VHL* tumor

suppressor gene [172-174]. PCC occurs in about 10-20% of vHL patients with about 5% of PCC cases diagnosed as malignant [174]. PCC in vHL are generally NE secreting and lack PNMT expression [170]. Multiple endocrine neoplasia type 2 (MEN2) is another autosomal dominant hereditary cancer susceptibility syndrome that presents most frequently with medullary thyroid carcinomas but PCC is detected in about 50% of patients (about 5% of these PCC are malignant) [164, 175, 176]. MEN2 is caused by activating mutations in the proto-oncogene *RET*, which encodes for the tyrosine kinase receptor c-Ret [177]. PCC identified in MEN2 patients frequently over express PNMT and as such, is predominantly epinephrine-secreting tumors [170]. The differential expression of catecholamines and their respective biosynthetic enzymes may suggest different chromaffin cells of origin (noradrenergic versus adrenergic) but may also reflect other less defined molecular changes specific to each syndrome.

Indeed, gene expression profiling of tumors from 12 vHL patients, 7 MEN2 patients, and 20 sporadic PCC, both epinephrine- and norepinephrine-secreting, has revealed common genetic perturbations specific to the predominant catecholamine secreted by the neoplasms analyzed [178]. Interestingly, there is enrichment of the Notch genes Notch3 and JAG1 in vHL and norepinephrine-secreting, sporadic tumors compared MEN2 and epinephrine-secreting, sporadic tumors and this may have implications for chromaffin cell differentiation (see below) [178]. Furthermore, downregulation of 8 genes associated with inhibition of Notch1 signaling (the specific genes were not identified) was identified in 13 malignant versus 45 benign PCC, which suggests inactivation of Notch signaling

may be involved in progression to a more aggressive PCC phenotype [179]. A similar observation was made in a study examining miRNA expression in 9 patients with sporadic benign PCC, 8 patients with benign MEN2 PCC, 6 patients with benign vHL PCC, and 5 with sporadic PCC that have recurred subsequent to surgical resection of the primary tumor [180]. The miRNA Mir-1225-3p was found to be upregulated in the sporadic, recurring PCC compared to benign tumors. Many of the predicted target mRNAs that Mir-1225-3p may inhibit were found to be Notch pathway genes, which imply a downregulation of these genes [180]. Taken together, these data indicate a differential activation of Notch signaling in PCC. The apparent decrease in Notch gene expression in malignant and recurring PCC suggests that Notch may be involved in conferring a more quiescent and/or differentiated PCC cell phenotype. Indeed, two studies have found that pharmacologic activation of Notch1 signaling through either histone deacetylase inhibitors or sodium butyrate inhibits proliferation of the PC-12 rat PCC cell line [181, 182].

In addition to PCC, the adrenal medulla is the origin of about 1/3 of pediatric neuroblastoma (NB) tumors [183]. NB presents in 1 in 700 children/year and is thus one of the most common childhood cancers, accounting for about 7-10% of cases/year, and is diagnosed in a majority of patients before 5-years of age [184]. The expression of genes normally expressed during development in NB suggests an embryonal origin derived from the SA progenitors arrested at various stages of differentiation [185-187]. The detection of c-Ret expression suggests a sympathetic neuronal precursor-like origin (opposed to a chromaffin

progenitor-like origin) of some NB [188]. In contrast to PCC, Notch1 expression has been correlated to less differentiated NB and to poor prognosis but comparison of expression differences between different types of NB was not made in this study [189]. Inhibition of Notch signaling of NB cells lines was also found to induce differentiation and retard proliferation of these cell lines [190]. However, the cell lines used in this study were not derived from NB of adrenal medullary origin. These data suggest Notch functions to maintain the undifferentiated state of neural precursors, the over activation of which may lead to NB [183]. Even though sympathethtic neuroblasts and chromaffin progenitors share a common lineage, contrasting functions of Notch signaling in NB and PCC suggest differential role of Notch signaling in the SA lineage.

Of particular interest in NB pathogenesis is the non-canonical Notch ligand Delta-like 1 (Dlk1/FA1/Pref1/ZOG). Dlk1 is a transmembrane protein structurally related to other canonical notch ligands; it contains EGF repeats but lacks the DSL domain [12, 191]. The role of Dlk1 in Notch signaling is currently unclear. In yeast 2-hybrid screens, it has been shown to interact with Notch receptors through its EGF repeats [192]. In cell culture, it is hypothesized to antagonize Notch receptors in c*is* (i.e. it inhibits Notch signaling in cell-autonomous manner) but this phenomenon has never been shown as consequence of direct interaction between Notch receptors and Dlk1 [193, 194]. In development, the function of Dlk1 in Notch signaling is far more ambiguous as suggestive by the expression of Dlk1 in numerous tissues where canonical Notch signaling is known to occur such as the placenta [195, 196], pancreas [197, 198], and lungs [199, 200]. The

identification of Dlk1 expression in chromaffin progenitors prior to invasion of the fetal adrenal gland, post-invasion, and in the adult gland suggests Dlk1 is a molecular marker of differentiated chromaffin cells [199, 201, 202]. Dlk1 expression has been robustly identified in a subset of NB cells lines concomitant with expression of Notch receptors Notch2 and Notch3 and Notch ligand JAG1 and catecholoamine biosynthetic enzyme DBH [202]. Furthermore, a comparison of differentially expressed genes in rat and human PCC identified Dlk1 as one of the genes upregulated in PCC from both species [203]. Taken together, these data suggest Notch signaling and Dlk1 expression may define a subset of NB as derived from SA progenitors committed towards a chromaffin cell fate. These data are in contrast to the putative role of Notch signaling in maintenance of a less differentiated sympathetic neural precursor in some NB but further research is required to define the precise function Notch in tumors of SA lineage.

Notch Signaling in the Development of the Neural Crest Lineage

The implication of the analysis of Notch in tumors of either adult or embryonal SA cells (PCC and NB respectively) is that Notch signaling may be involved in the development of this lineage. Notch1, 2 and 3 appear to be expressed in non-overlappping patterns in early neurulation and in formation of the NC while Notch1 appears to be restricted to NC cells of trunk region, precurors to SA progenitors [204]. In the quail embryo, DLL1 expression is

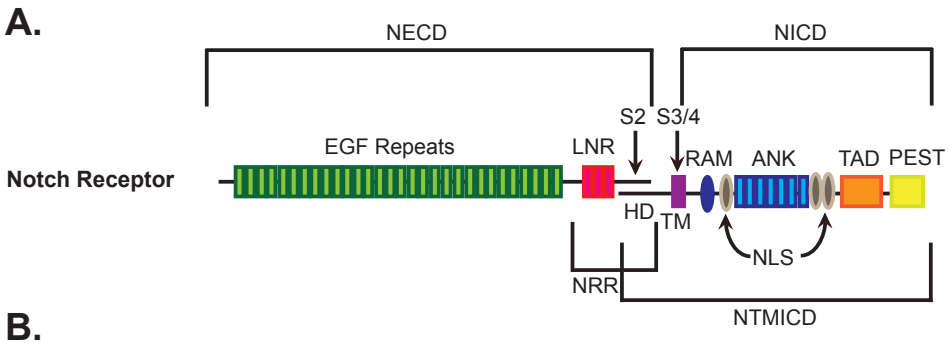
restricted to the neural ectoderm prior to NC formation while Notch1 exhibited a broad expression pattern throughout the neural fold [205]. Notch1 overactivation inhibited NC formation as indicated by loss of the transcription factor Slug, believed to be involved in epithelial-to-mesenchymal transition of the neural ectoderm in NC initiation, while inhibition of DLL1 had the opposite effect [205]. BMP4, a putative signal in initiation of NC formation, was modestly affected in these studies [142, 205]. Taken together, these data suggest that Notch signaling may participate in initiation of NC formation through transient induction and/or maintenance of BMP4 expression. Two similar studies in zebrafish have provided additional evidence supporting the function of Notch in specification of the NC versus spinal sensory neurons [206, 207].

In addition to its putative role in initiation of development of the NC, Notch signaling has been shown to be involved in NC cells later in development. In heart development, canonical Notch signaling was inhibited in Pax3-positive cells, a marker of cardiac NC cells, which resulted in a wide range of cardiovascular abnormalities [208]. Migration of cardiac NC cells was reported as unaffected in these mice. However, no effect on SA progenitors and the adult tissues they form was reported which is not surprising since cardiac NC cells are considered distinct from the NC cells that give rise to SA progenitors [132, 208].

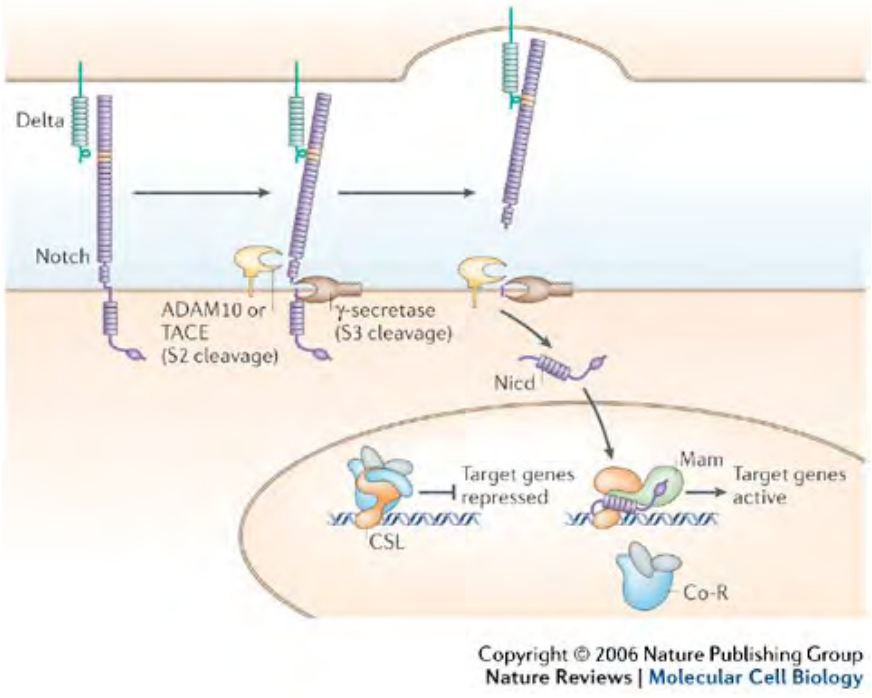
In sympathetic ganglia of the developing chick embryo, DLL1, Notch1, and Hes5 expression was detected in early in development of sympathetic ganglia but this expression was severely reduced at later embryonic time points [209]. When Notch signaling was constitutively activated in developing sympathetic

ganglia, a reduction in the number of SCG10-positive neurons was observed while the opposite was observed with the introduction of a dominant-negative inhibitor of Notch transcription [209]. These data suggest Notch may suppress differentiation of sympathetic neurons from SA progenitors. No evidence on whether Notch may drive SA progenitors towards a chromaffin cell fate was provided in this study but the common lineage between the two cell populations, including expression of several SA-specific transcription factors, presupposes this possibility. As previously discussed, the nan-canonical Notch ligand Dlk1 is expressed in chromaffin progenitor pre- and post-migration into the adrenal anlage [199, 201, 202]. However, no further characterization of Notch signaling in chromaffin cell progenitors or adult chromaffin cells have been performed. Chapter 3 of this thesis addresses the role of Notch signaling in the development and function of the adrenal gland with emphasis on chromaffin cells of the adrenal medulla.

Figure 1.1: The Notch Signaling Pathway. A. Basic structure of Notch receptors. NECD: Notch extracellular domain, NTMICD: Notch transmembrane/intracellular domain, NICD: cleaved Notch intracellular domain, NRR: negative regulatory region, LNR: cysteine-rich LIN12-Notch repeats, HD: heterodimerization domain, TM: transmembrane domain, RAM: RBPjκ association module, ANK: ankyrin repeats, TAD: transactivation domain, NLS: nuclear localization signals, PEST: proline/glutamic acid/serine/threonine-rich domain S2, S3/4 cleavage sites are indicated with arrows **B.** DSL: delta/serrate/lag-2 domain, DOS: Delta and OSM-11-like protein domain, CR: cysteine-rich region, TM: transmembrane domain. **C.** Overview of the basic Notch signaling. Ligands and receptors expressed on adjacent cells interact through their extracellular domain. Upon Ligand/receptor transendocytosis, the S2 site is exposed and cleaved by ADAM metalloproteases. The γ-secretase complex then cleaves at S3/4 site, which results in release of the NICD. The NICD transits to the nucleus where it interacts with DNA-bound CSL protein and recruits the transcriptional co-activator Mam (Maml in vertebrates) to initiate Notch-dependent transcription.



C.



(Adapted from Bray S. *Nat. Rev. Mol. Cell Biol.* 2006. 7:678-689.)

Figure 1.2: Biology of the Adrenal Gland. A. Endocrinology of the Hypothalamic-pituitary-adrenal axis. In response to stress, corticotropin releasing factor (CRF) released from the hypothalamus acts on coricotropes of the anterior pituitary gland to stimulate release of adrenocorticotropic hormone (ACTH). ACTH stimulates secretion of cortisol from the adrenal cortex, which flows through the blood stream to induce systemic metabolic effects in addition to regulating the secretion of CRF and ACTH though a negative-feedback loop. **B.** The discrete zones of the adrenal gland and their respective hormone secretions. The adrenal cortex is marked by expression of the nuclear receptor steroidogenic factor 1 (Sf1), is enclosed by a mesenchymal capsule, and is composed of three concentric zones. The outer Zona Glomerulosa secretes mineralocorticoid hormones such as aldosterone. The middle Zona Fasiculata produces the glucocorticoid hormone cortisol (corticosterone in mice). The inner Zona reticularis (not present in mice) secretes sex steroid precursors such as dihydroepiandosterone (DHEA) and DHEA-sulfate (DHEA-S). The inner medulla, marked by tyrosine hydroxylase (TH) expression and of distinct ontogeny from the adrenal cortex, secretes catecholamine hormones epinephrine (E) and, to a lesser extent, norepinephrine (NE).

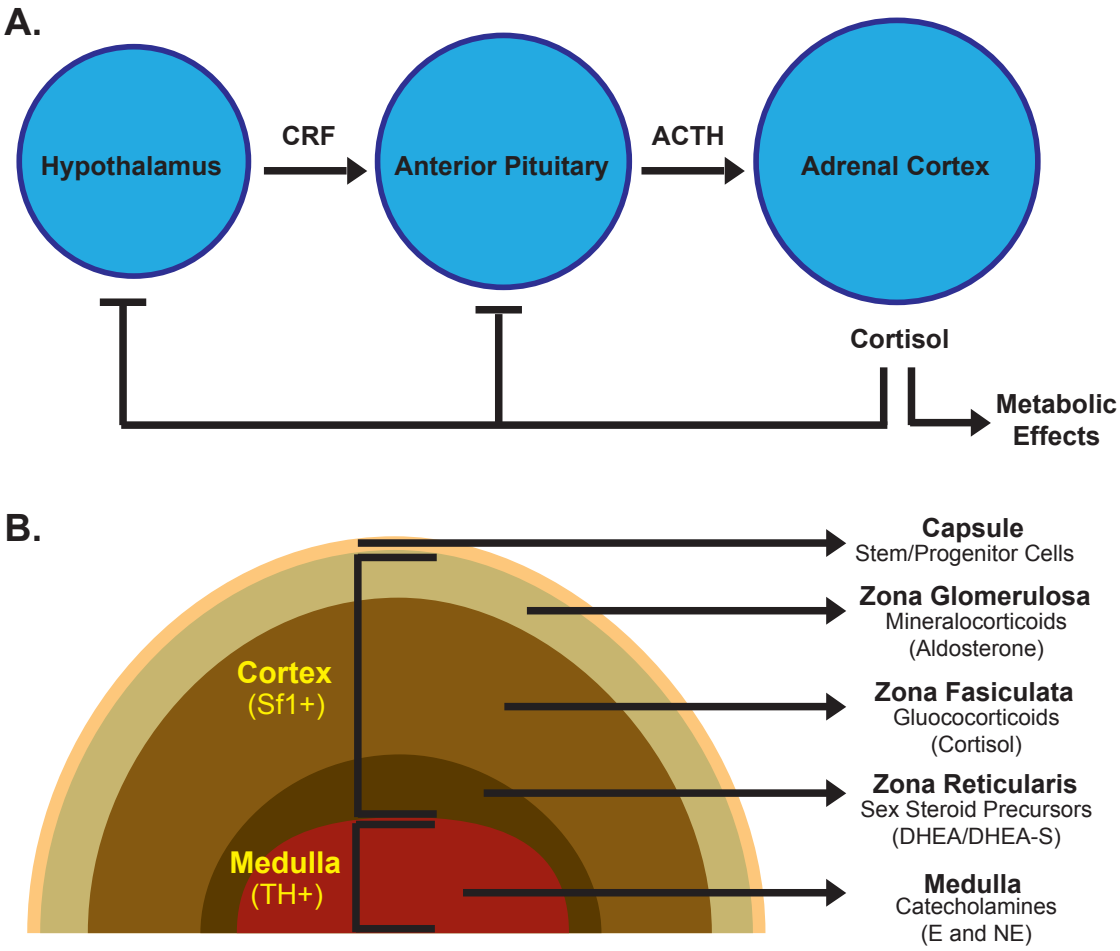
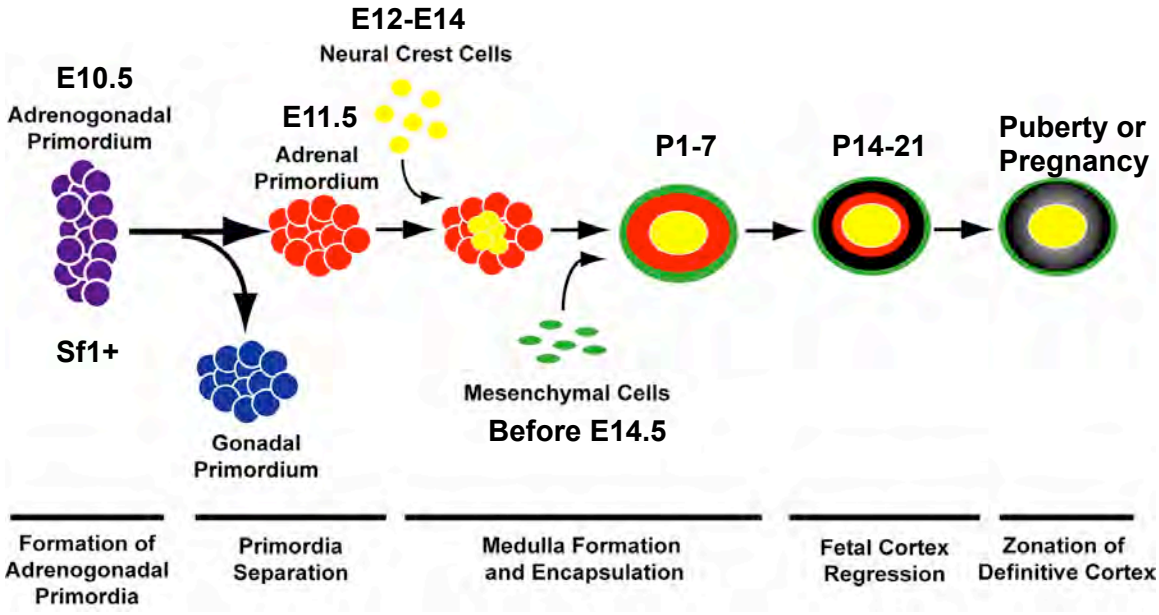

A.
Hypothalamus
CRF
Anterior Pituitary
ACTH
Adrenal Cortex
Cortisol
Metabolic Effects
B.
Cortex
(Sf1+)
Medulla
(TH+)
Capsule
Stem/Progenitor Cells
Zona Glomerulosa
Mineralocorticoids
(Aldosterone)
Zona Fasiculata
Gluococorticoids
(Cortisol)
Zona Reticularis
Sex Steroid Precursors
(DHEA/DHEA-S)
Medulla
Catecholamines
(E and NE)

Figure 1.3: Development of the Adrenal Gland. Formation of the bipotential adrenogonadal primordium (AGP) is initiated by expression of steroidogenic factor 1 (Sf1) expression around E10.5 in mice. The AGP bifurcates into the adrenal and gonadal primordial at approximately E11.5. Invasion of the fetal adrenal gland (adrenal primordia) by chromaffin progenitor cells begins at E12 and ceases around E14. This is concurrent with encapsulation of the adrenal cortex by mesenchymal cells. The definitive (adult) cortex begins to develop shortly after birth concomitant with the regression of the fetal adrenal and is complete by about P21. A residual fetal (X-zone) persists until puberty in males and after the first pregnancy in females.



(Adapted from Wood M and Hammer GD. *Mol. Cell Endocrinol.* 2011. 336:206-212.)

Figure 1.4: Adrenocortical Tumorigenesis. Adrenocortical adenomas (ACA) are benign tumors that are common, benign, well differentiated, and generally encapsulated. Overactivation of the Wnt signaling pathway through of variety of mechanisms has been implicated in ACA formation. Adrenocorticacarcinomas (ACC) are rare tumors but are highly aggressive and frequently fatal. ACC are composed of heterogenous population of cell types, and are highly invasive and vascularized, and frequently have metastasized upon detection. Overactivation of the Insulin-like growth factor (IGF) pathway, especially upregulation of IGF2, concurrent with overactivation of the Wnt pathway has been posited as critical genetic hits in ACC formation. The status of the Notch pathway is unknown in ACC.

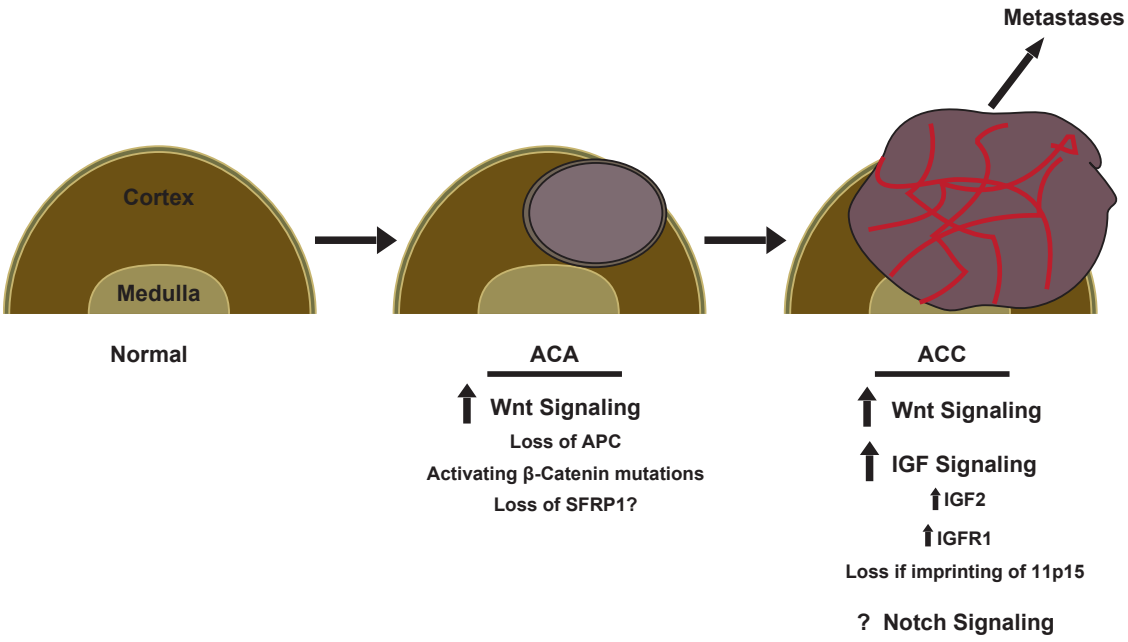
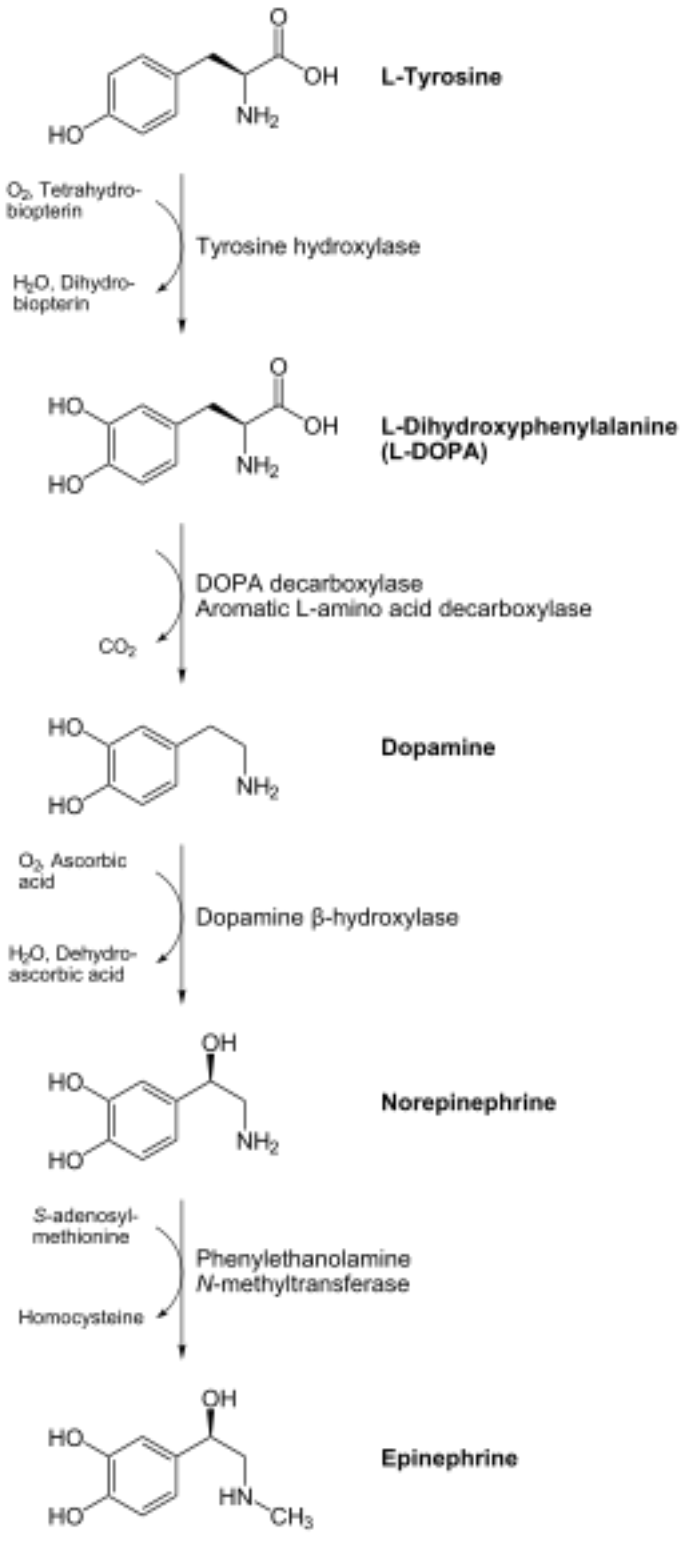

Metastases
Cortex
Medulla
Normal
ACA
Wnt Signaling
Loss of APC
Activating β-Catenin mutations
Loss of SFRP1?
ACC
Wnt Signaling
IGF Signaling
IGF2
IGFR1
Loss if imprinting of 11p15
? Notch Signaling

Figure 1.5: Biosynthesis of Catecholamines. Catecholamine hormones are derivatives of the amino acid L-tyrosine, which is enzymatically converted to L-DOPA by the critical, rate-limiting enzyme tyrosine hydroxylase, expressed in all chromaffin cells of the adrenal medulla. L-DOPA is converted to Dopamine by aromatic L-amino acid decarboxylase (DOPA decarboxylase), which is subsequently converted to norepinephrine by dopamine-β-hydroxylase. In the adrenal medulla, norepinephrine is converted to epinephrine by phenylethanolamine-N-methyltransferase but only in chromaffin cells that express this enzyme.



(Adapted from www.wikipedia.com)

Figure 1.6: Basic Molecular Biology of Chromaffin Cells of the Adrenal Medulla. A. Direct innervation of the adrenal medulla is through the thoracic splanchnic nerve, which releases the acetylcholine (ACh) and pituitary adenylate cyclase-activating protein (PACAP) and induces secretion of the catecholamine hormones epinephrine and norepinephrine during the "fight or flight" stress response. **B.** Important molecules in adrenergic chromaffin cell biology. Adrenergic chromaffin cells express the enzyme PNMT that is required for the conversion of norepinephrine to epinephrine (E). Catecholamines are transported into chromaffin granules through vesicular monoamine transporters (VMAT1 is the predominant VMAT in chromaffin cells). Peptide hormones chromogranin A (ChrA) and neuropeptide Y (NPY) are concurrently packaged into chromaffin granules. Granule exocytosis and release of the enclosed hormones is mediated through splanchninc nerve mediated release of ACh and PACAP, which bind to nicotinic acetylcholine receptors (NAChR) and PAC1, respectively. Cessation of the "fight or flight" induced catecholamine release is partly mediated through reuptake of catecholamines back into chromaffin cells through the solute carrier family of plasma membrane transporters that include dopamine transporter 1 (DAT1) and noradrenaline transporter 1 (NAT1). NPY released with catecholamines can also feedback onto chromaffin cells through the NPY Y_1 receptor.

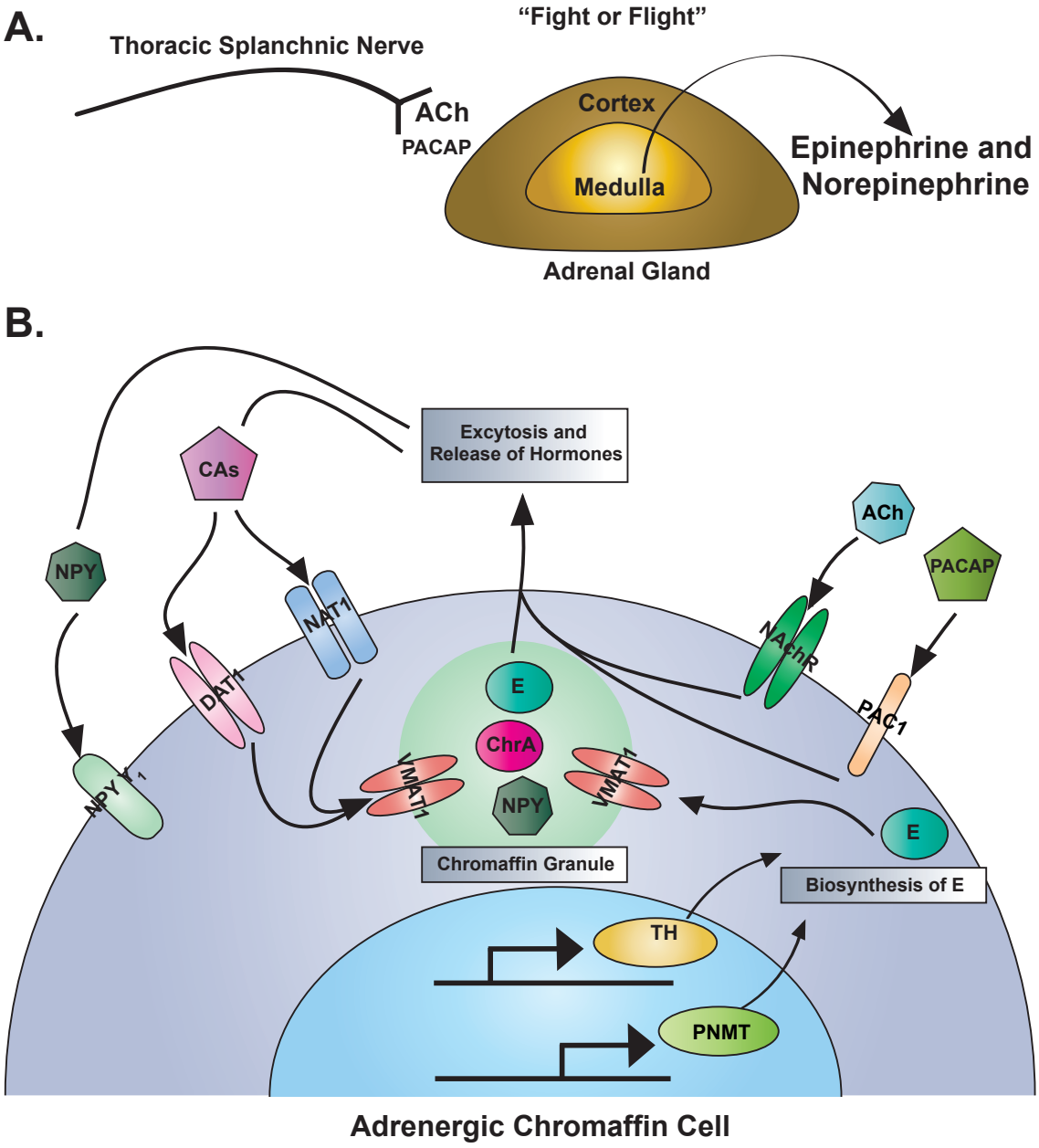
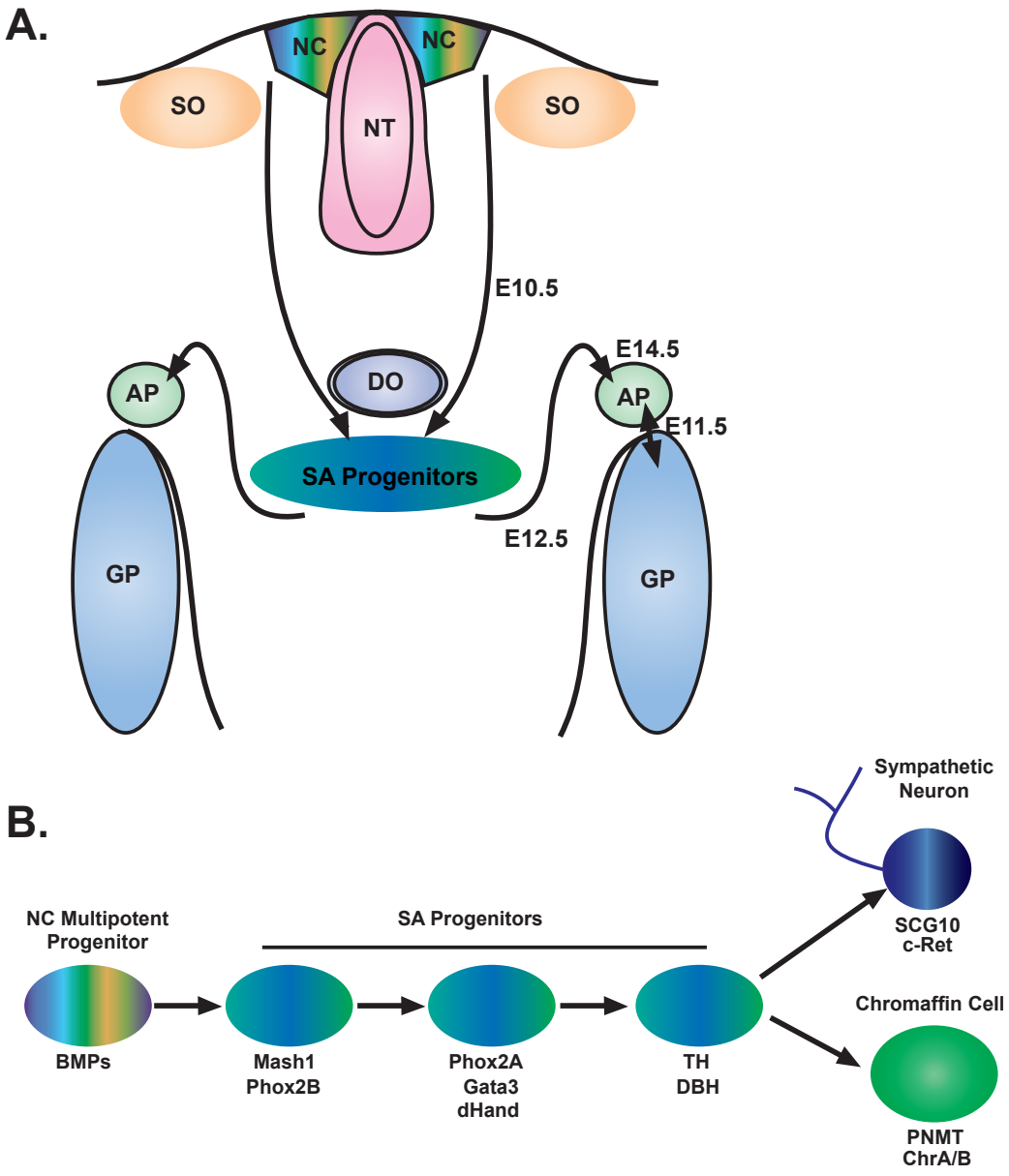

A.
Thoracic Splanchnic Nerve
ACh
PACAP
"Fight or Flight"
Cortex
Medulla
Epinephrine and
Norepinephrine
Adrenal Gland
B.
CAs
Excytosis and
Release of Hormones
NPY
ACh
PACAP
NAT1
DAT1
NAChR
PAC1
E
ChrA
NPY
VMAT1
VMAT1
NPY 1
Chromaffin Granule
E
Biosynthesis of E
TH
PNMT
Adrenergic Chromaffin Cell

Figure 1.7: Development of Chromaffin Cells. A. Migration of SA progenitors into the adrenal anlage. Multipotent neural crest (NC) progenitors arise from the dorsolateral sides of the neural tube (NT) post neurulation. Shortly after, NC cells of the sympathoadrenal (SA) lineage begin to migrate between the somites (SO) and receive inductive signals in transit and from the dorsal aorta (DO), committing them towards a SA progenitor fate. Migration and initial differentiation of SA progenitors is complete by about E10.5 in mice. After separation of the adrenal primordia (AP) and gonadal primordial (GP) (about E11.5 in mice), SA progenitors that are destined to become chromaffin cells begin to migrate into the AP (E12.5 in mice), which is complete by E14.5. Independent of chromaffin progenitors into the AP, other SA progenitors migrate and form sympathetic ganglia (not shown). **B.** Maturation of cells of the SA lineage. Commitment of multipotent NC progenitors towards the SA lineage is initiated through bone morphogenic protein (BMP) signals; BMPs may also initiate migration of SA progenitors to the DO. The SA lineage is defined by expression of the transcription factors Mash1 and Phox2B, which induces the expression of the downstream transcription factors Phox2A, Gata3, and dHand. Ultimately, SA progenitors express TH and dopamine-β-hydroxylase (DBH), enzymes common to both cells of the sympathetic nervous system, adrenal medulla, and extra-adrenal paraganglia. In a poorly understood process, SA progenitors are committed towards either a sympathetic neuronal or chromaffin cell fate. Downregulation of neuronal markers SCG10 and c-RET, as well as induction of chromogranin A and B (ChrA/B) and the epinephrine-synthesizing enzyme PNMT,

are molecular hallmarks of mature, adrenergic chromaffin cells (Noradrenergic chromaffin cells proceed are marked by the same molecular hallmarks but lack PNMT expression).



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Chapter 2: The Role of Notch Signaling in Adrenal Cancer*

Abstract

Adrenocortical adenomas (ACA) are common (~4% of the population) benign neoplasms of the adrenal gland while adrenocortical carcinomas (ACC) are rare (~0.5-2 cases/million) highly malignant cancers. Due to difficulty of detection and lack of effective treatments, ACC frequently presents with an extremely poor prognosis. Despite recent technological advancements in genetic profiling of ACC, the molecular pathogenesis of ACC has remained unclear, particularly pertaining to factors involved in late-stage disease. Interrogation of our recent adrenocortical tumor cDNA expression array data set identified an upregulation of the Notch ligand JAG1 in ACC compared to ACA and normal adrenals. Notch signaling is a highly conserved developmental signaling pathway that involves interaction between a transmembrane ligand (JAG1) and its cognate receptor (Notch) expressed on adjacent cells. To interrogate the role of JAG1 in ACC, the Y1 mouse ACC cell line was identified as an ACC cell line that exhibits JAG1 activated Notch signaling. Experiments employing a Jag1 knockdown strategy

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and utilizing a co-culture system with FACS analysis reveal a non-cell autonomous inhibition of proliferation in the Y1 cell line. Furthermore, inhibition of Notch-dependent transcription phenocopies the Jag1 knockdown. Lastly, JAG1 is positively correlated with both advanced stage and mitotic rate in patients with ACC in addition to markers of proliferation KI67 and TOP2A. Taken together, the data indicate that JAG1 augments cell proliferation in a non-cell-autonomous manner, consistent with a role of the Notch pathway activation in ACC initiation, maintenance or progression. JAG1 and the Notch signaling pathway may be novel targets for therapeutic intervention in late-stage ACC.

Introduction

Adrenocortical Tumors (ACT) are extremely common neoplasms, the vast majority being benign adrenocortical adenomas (ACA) that occur in as many as 4-7% of the population whereas adrenocortical carcinomas (ACC) are extremely rare (~0.5-2 cases/million) accounting for 0.2% of cancer deaths annually [1, 2]. ACC is typically an aggressive neoplasm with many patients presenting with metastases upon diagnosis [1]. Due to difficulty of early detection and lack of effective treatments for advanced-stage ACC, the average survival for surgically unresectable tumors is 12-months and the overall 5 year survival is historically less than 10% [3, 4]. The molecular pathogenesis of ACC has remained elusive until recently. Dysregulation of developmental signal transduction pathways is found in an increasing number of cancers including ACC. Specifically, the Wnt

signaling pathway, a critical mediator of adrenal development, plays an important role in the etiology of ACC, where constitutively active, nuclear β-catenin is frequently observed [5-9]. The development of visible adrenal tumors in mice engineered to express constitutively-active β-catenin in the mouse adrenal cortex supports the hypothesis that dysregulation of Wnt/β-catenin signaling is a vital step in adrenocortical tumorigenesis [10].

Similar to the Wnt pathway, Notch signaling is involved in a wide range of cell fate decisions during development. While its dysregulation is a common molecular event in a variety of cancers, its role in adrenal development and ACC is unknown [11, 12]. Notch signaling involves interaction between a transmembrane ligand, of either the Jagged (JAG1/2) or Delta-like (DLL1/3/4) family, and a transmembrane receptor (NOTCH1/2/3/4) generally expressed on adjacent cells [13, 14]. Upon binding of Notch ligand to receptor, the γ-secretase complex cleaves the Notch receptor in two locations releasing the active signaling molecule NICD (cleaved Notch intracellular domain). NICD interacts with constitutively DNA-bound CSL (CBF-1/RBPjκ/Su(H)/Lag-1), recruits the essential transcriptional coactivator MAML (Mastermind-like) and initiates transcription of Notch-dependent genes such as the HES (hairy enhancer of split) family of transcription factors.

The upregulation of the Notch ligand, Jagged1 (JAG1), in a variety of cancers implies a ligand-dependent activation of the Notch signaling pathway [15-19]. Indeed, the upregulation of JAG1 in breast and prostate cancer has been implicated in metastatic disease and correlates with poor prognosis [20-23].

Mechanistically, JAG1 is thought to enhance the metastatic potential of breast cancer through a Notch-dependent induction of epithelial-to-mesenchymal transition of mammary epithelial cells [24]. Like all Notch ligands, JAG1 classically interacts with receptors on adjacent cells (non-cell-autonomous) rather than with receptors on the cells in which they are expressed (cell-autonomous). However, the ability of JAG1 to induce malignant transformation of RKE cells despite the absence of Notch receptors raises the possibility that non-canonical actions of JAG1 mediate some of its oncogenic manifestations [25]. In this study, we report for the first time that JAG1 is the primary upregulated Notch ligand in ACC and enhances ACC cell proliferation and tumor aggressiveness in a non-cell-autonomous manner through activation of Notch signaling in adjacent cells.

Materials and Methods

Microarray Analysis

DNA Microarray analyses were performed with Affymetrix U133A 2.0 Plus oligonucleotide arrays and have been published [26, 27]. Probe sets for JAG1, JAG2, DLL1, DLL3, and DLL4 were presented in a heatmap with clustering delineated by tumor type; individual samples were ordered based on JAG1 expression as determined by probe sets 216268_s_at and 209099_x_at. For a complete list of probe sets of Notch pathway genes used in dot plots and correlations (**Fig 2.1-2.3**), see **Table 2.1**. Additional correlations were performed

using JAG1 probe set 216268_s_at with KI67 212022_s_at, and with TOP2A 201292_at. Similar correlations were obtained with other JAG1 probe sets.

Human Samples

Protein and RNA were extracted using routine protocols from frozen adrenocortical tissues obtained via the University of Michigan Comprehensive Cancer Center Tissue Procurement Service with IRB approval. Samples for protein and RNA analysis were randomly selected [normal adrenal (NL): n=5, ACA: n=5, ACC: n=10]. Due to tissue availability, different pools of samples were analyzed for message and protein.

Plasmids, shRNA and transfection

Notch reporter (pJH23A: 4xwtCBF1Luc) and Control Reporter (pJH25A: 4xmtCBF1Luc) expression vectors were a generous gift from Dr. S. Dianne Hayward (John Hopkins University Medical School, Baltimore, MD) [28]. The Notch reporter contains four consensus CSL binding sites driving expression of firefly luciferase while these sites are mutated in the Control Reporter. pGIPZ vectors (Open Biosystems, Huntsville, AL) expressing shRNA against JAG1 and a non-specific scrambled control shRNA (Scramble) were obtained from the University of Michigan shRNA core (http://fgc.lsi.umich.edu/index.html). In addition to the shRNA, pGIPZ vectors contain a puromycin selection cassette

and an IRES GFP sequence. Sequences for JAG1 shRNA are #1: 5'-gtcagaattgtgacataaa-3' and #2: 5-gggatttggttaatggtta-3'. pdsREDII expresses dsREDII under control of the CMV promoter and was obtained from Dr. Claudius Vincenz (University of Michigan, Ann Arbor, MI). Control (MigR1) plasmid, which expresses GFP, and DNMaml plasmid, which expresses a fusion protein of GFP and amino acids 13-74 of Maml1 and acts as a dominant-negative, were a generous gift of Dr. Ivan Maillard (University of Michigan) [29, 30]. Both Control (MigR1) and DNMaml plasmids contain flanking LTR sequences and expression is driven by an MSCV promoter. Retroviral packaging protein expression plasmids pGag/Pol and pVSV were kindly provided by Dr. Michael Malim (King's College, London, UK).

Cell Culture and Generation of Stable Cell Lines

Culture of the mouse ACC cell line Y1 [31] and the Human ACC cell lines NCI-H295A [32] and RL251 [33] has been described previously [27, 34]. All standard cell culture reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). MOLT4 T-ALL cell line (Item # CRL-1582) was obtained through American Type Culture Collection (ATCC, Manassas, VA). Virus-competent 293T cells, a gift from Dr. Benjamin Margolis (University of Michigan), were maintained in DMEM with 10% Cosmic Calf Serum (CCS, Hyclone, Logan, UT) and penicillin/streptomycin. In some experiments, Y1 cells were treated for 6h with 5mM EDTA prepared in PBS. Transient transfections were performed using

Fugene (Roche, Madison, WI) according to the manufacturer's instructions and optimized at a 4:1 ratio (4µl Fugene/1µg DNA) for Y1 cells and 2:1 ratio for 293T cells.

For generation of Scramble (GFP+) and Jag1KD (GFP+) stable cell lines, Y1 cells were transfected with 2µg of pGIPZ vectors expressing shRNA directed against Jag1 or a control (Scramble) as described above, followed by 4 weeks of puromycin selection (2µg/ml, Roche). Cells were then enriched for GFP expression within the 10^4-10^5 range using Fluorescence Activated Cell Sorting (FACS) as described below. Sorted cells were replated in 10cm dishes and allowed to expand.

To obtain the dsREDII [wildtype Y1 (Red+)] cell line, 10cm dishes of Y1 cells were transiently transfected with 2µg of pdsREDII as described. Because pdsREDII lacks a mammalian selection cassette, cells were passaged after 2 days and were transiently transfected an additional time. After 2 days enriched for with high dsREDII expression within the 10^4-10^5 range by FACS. Sorted cells were replated in 10cm dishes and allowed to expand.

To generate the Control (GFP+) and DNMaml (GFP+) stable cell lines, viral supernatant was generated by contransfection of 293T cells with 2µg each of pGag/Pol, pVSV, and either Control (MigR1) or DNMaml constructs. After 2 days, medium was collected and centrifuged at 5,000xg followed by filtration through a 0.22µM syringe nylon filter (Fisher Scientific, Pittsburgh, PA). Viral supernatant was adjusted to a final volume of 10ml with DMEM and polybrene

was added (10U/ml, Sigma, St. Louis, MO). Y1 cells were then transducted with viral supernatant for 24 hours. Cells were passaged and transducted an additional time under identical conditions. Cells were then enriched for GFP expression within 10^4-10^5 range using FACS and were replated into 10cm dishes and allowed to expand. Because the GFP and dsREDII expression diminished overtime, cells were resorted under identical parameters every 3 months.

Fluorescence Activated Cell Sorting and Analysis

Trypsinized cells were pelleted at 1,000xg for 5min and resuspended in 1X PBS containing 10% CCS at a concentration of 1-2million cells/ml. FACS experiments were performed by the University of Michigan Flow Cytometry Core (http://www.med.umich.edu/flowcytometry/) with either BD Biosciences FACSDiVa High-Speed Cell Sorter (3-laser: 488nm, 350nm and 633nm) or BD Biosciences FACSAria High-Speed Cell Sorter (3-laser: 488nm, 407nm and 633nm).

Quantiative real time-PCR (QPCR) Analysis

RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) according the manufacturer's instructions and cDNA was generated using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). QPCR experiments were performed as previously described [35, 36]. A comprehensive list of human and

mouse QPCR primers is found in **Table 2.2**. Analysis was conducted with either the efficiency-corrected ΔCT method or the ΔΔCT method as indicated [37]. Expression of mRNA was normalized to β-actin.

Immunocytochemistry and Immunoblots

For a comprehensive list of primary and secondary antibodies used for Immunocytochemistry (ICC) and Immunoblots see **Table 2.3**. For immunocytochemical localization: Y1 cells were plated on glass slides coated with fibronectin (10μg/ml, Sigma). Slides were washed in 1X PBS, fixed in 4% paraformaldehyde (Fisher) for 15min at 4°C, and permeabilized with 0.02% Igepal CA-630 (Sigma). Slides were blocked with 2% milk in 1X PBS and primary/secondary antibodies (**Table 3.3**) were diluted in 0.2% milk in 1X PBS. For detection of native fluorescence, slides were not fixed in order to preserve the activity of GFP and dsRedII. Cover slips were applied and images obtained as previously described [35, 36].

Immunoblot analysis of protein lysates from cell cultures were performed as previously described[35]. Analysis of some protein lysate was conducted as described but blocking, primary, and secondary dilutions were done in Odyssey Blocking buffer (LICOR, Lincoln, NE), secondary antibodies used were Odyssey IRdyes (**Table 3.3**). Immunoblots using protein lysate from human adrenal tumor samples was quantified using ImageJ software (National Institutes of Health, Bethesda, Md). The btan20 (Notch1) and C651.6DbHN (Notch2) monoclonal

antibodies were developed by Spyros Artavanis-Tsakonas and were obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, 52242.

MTS Proliferation Assay

The MTS cell viability assay translates cell number into a colorimetric readout (absorbance) via metabolic breakdown of tetrazolium salts (Promega Corp, Madison, WI). Cells were plated in 96-well plates and assay performed according to the manufacturer's instructions. Absorbance values were obtained using a SpectraMAX190 plate reader (Molecular Devices, Sunnyvale, CA).

Co-culture Experiments

Stable cell lines of Y1 cells expressing dsRedII [wildtype Y1 (Red+)] were co-cultured with either Scramble (GFP+) or Jag1KD (GFP+) Y1 cells in triplicate wells of a 6-well plate in two ratios: 90% Red+/ 10% GFP+ and 10% Red+/ 90% GFP+. The combined initial concentration for each ratio at each time point was 150,000 cells/well. Cells analyzed at the Day 4 time point were plated 4 days in advance of analysis, Day 3 time point were plated 3 days in advance of analysis, etc. At the end of the 4-day time course, harvested cells were analyzed by FACS as described above. 10,000 cells were analyzed for each sample and the cell

number for each color (Red+ or GFP+) was determined and data are presented as a percentage change from Day 1 (For schematic, Fig 4A). Identical conditions and analysis were performed for co-culture of wildtype Y1 (Red+) with either Control (GFP+) or DNMaml (GFP+) except that a 50%/ 50% ratio was used.

Luciferase Assays

Y1 cells were plated in 24-well plates and were transiently transfected with 50ng of pRL-TK *Renilla* Luciferase (Promega Corp, Madison, WI) and 0.5µg of either Control Reporter (pJH25A) or Notch Reporter (pJH23A) firefly luciferase constructs described above. Assays were performed 24h after transfection using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturers instructions and optimized for Y1 cells. Cells were lysed in 1X Passive Lysis Buffer and lysates analyzed on the Glomax Multi-detection System (Promega). Expression was normalized to pRL-TK *Renilla* Luciferase.

Pharmacologic Inhibition of Notch Signaling

Y1 and MOLT4 cells were plated in 6-well plates and were treated with either vehicle (DMSO) or 100µM of DAPT (D5942, Sigma) for 24h. Cells were harvested for protein and mRNA analysis. Y1 cells were plated in 6-well plates and were treated with vehicle (DMSO) or 100, 250, 400, 500ng of Compound E (γ-secretase inhibitor-XXI, Cat #: 565790, CEMD Biosciences Inc., San Diego,

CA). Cells were harvested after 24h for protein and mRNA analysis. Inhibitory Notch antibodies were acquired from Genentech (San Francisco, CA) and have been described previously [38]. Y1 cells were plated in 24-well plates and treated with Vehicle (PBS) or 80ng of either anti-Notch1 (YW169.60.79, PUR20635), anti-Notch2 (YW163.54.76, PUR23852) or a combination of both for 4h or 24h. Cells were harvested for mRNA analysis.

Statistics

All comparisons made used the student's T-Test. Statistical analysis of microarrays has been described elsewhere [26, 39].

Results

JAG1 is Upregulated in Human ACC.

In an effort to better understand the molecular characteristics of human ACC, our group has previously performed DNA microarray analyses using frozen human tissues – most recently with a total of 33 ACC, 22 ACA, and 10 normal adrenals (NL) [26, 39]. Analysis of differentially-expressed probe sets revealed an upregulation of the Notch ligand JAG1 in ACC samples compared to normal and adenomatous tissue (**Fig 2.1A**). The five JAG1 probes sets depicted are within the top 0.8% of all rank-ordered upregulated probe sets represented in the

microarray. The other four Notch ligands (JAG2, DLL1/3/4) were upregulated in only a few ACC.

Quantitative analysis of two independent probe sets for each JAG ligand (JAG1 and JAG2) confirmed that JAG1 expression is significantly higher in ACC samples compared to NL and ACA (Top panels, **Fig 2.1B**). JAG2 exhibits a statistically significant, albeit less dramatic, difference in expression among samples (Bottom panels, **Fig 2.1B**). Furthermore, interrogating two other adrenal tumor microarray data sets revealed a similar upregulation of JAG1 in ACC[39, 40].

Microarray analyses were validated with QPCR of mRNA from human adrenal tumor samples (**Fig 2.1C**). Correlation of QPCR and microarray data for each samples is shown in **Fig 2.1D**. While both JAG1 and JAG2 were significantly different in ACC vs ACA/NL, JAG1 is expressed at a higher level and a greater increase in ACC than JAG2. Additionally, JAG1 QPCR expression was more tightly correlated to the microarray data (JAG1: r=0.874, JAG2=0.545). These data support the validity and biological relevance of the microarray results.

Furthermore, immunoblot analysis of human adrenal tumor samples revealed a higher expression of JAG1 protein in the majority of ACC when compared to NL and ACA (Top panel, **Fig 2.1E**). Quantification of band intensity of two immunoblots using the same set of human samples identifies robust protein levels of JAG1 in most ACC and barely detectable quantities in ACA and NL (Bottom panel, **Fig 2.1E**). Together these data suggest that JAG1 mRNA and

protein is upregulated in a majority of ACC samples and is consistent with biological relevance of JAG1-activated Notch signaling contributing to adrenocortical carcinogenesis. While JAG2 is also upregulated, it exhibits a lower level of expression and a poorer correlation of QPCR and array data. Therefore, we decided to focus exclusively on JAG1, the significance of its upregulation in ACC, and its role in adrenocortical carcinogenesis.

Notch Receptors and Target Genes Exhibit Variable Expression in ACC.

Since JAG1 is hypothesized to mediate its functions through interaction with Notch receptors, the expression of Notch receptors (Notch1-4) was analyzed in our microarray data set (**Fig 2.2**). Quantitative analysis of dot plots of two independent probe sets for Notch1-3 and one probe set for Notch4 revealed no significant change in expression ACC compared to ACA and NL for a majority of the probe sets analyzed. However, Notch2 Probe Set #1 did exhibit a modest albeit significant upregulation in ACC compared to NL and in ACA vs NL (**Fig 2.2A**) but no positive correlation to JAG1 expression was identified (**Fig 2.2B**, top panel). Microarray analysis of Notch2 expression was validated with QPCR of mRNA from human adrenal tumor samples in which many ACC samples exhibited higher mRNA expression of Notch2 compared to benign ACA and NL adrenals (**Fig 2.2C**). Interestingly, some ACA samples did show increased Notch2 expression. Similarly, Notch3 Probe Set #2 exhibited a statistically significant increase in expression in ACC compared to ACA and NL. We also

identified a modest, statistically significant, positive correlation between NOTCH3 and JAG1 expression (r=0.29, p=0.02, **Fig 2.2B**, bottom panel). These data reveal that Notch2 and Notch3 are upregulated in ACC while Notch1 and Notch4 are not. However, the lack of strong correlations with JAG1 expression suggests that a concurrent upregulation of Notch receptors may not be required to facilitate augmented Notch ligand-receptor signaling in ACC.

Next, we considered the expression of Notch target genes that have been confirmed targets in numerous systems, Hes1 and Hey1 (**Fig 2.3**) [11, 13]. Quantitative analysis of dot plots of two independent probe sets for Hes1 and Hey1 revealed a significant change in expression of Hey1 in ACC compared to ACA and NL for both probe sets but no significant changes for either Hes1 probe set (**Fig 2.3A**). Both Hey1 probe sets also exhibited positive correlations with JAG1 expression (Probe Set #1: r=0.34, p=0.0053, Probe Set #2: r=0.34, p=0.0054) (**Fig 2.3B**). QPCR of mRNA from human adrenal tumor samples validated the upregulation of Hey1 in ACC (**Fig 2.3C**, bottom panel). In contrast with the microarray data, elevated mRNA of Hes1 was also detected in both ACA and ACC samples (**Fig 2.3C**, top panel). These data reveal that Notch target genes are significantly upregulated in ACC although numerous other targets not considered in our analysis may be regulated by Notch signaling in adrenocortical tumorigenesis.

The Y1 Mouse ACC Cell Line Exhibits Active Notch Signaling

Predicated on the canonical role of JAG1 as an obligate ligand for Notch activation, normal mouse adrenal (Adrenal), the mouse ACC cell line (Y1), and the two cell lines derived from human ACC (H295A and RL251) were surveyed for concurrent Jag1 abundance and expression of the Notch signaling components. Jag1 protein is highly expressed in both Y1 and H295A lines, recapitulating in an *in vitro* context the upregulation of JAG1 observed in the human ACC samples (**Fig 2.4A**), with Jag1 mRNA showing a 43-fold increase over Jag2 and Dll1/3/4 ligands being barely detectable (**Fig 2.4B**). This latter comparison demonstrates that the Y1 cell recapitulates the Notch ligand expression profile observed in the human ACC microarray where the expression of the other four Notch ligands is only modestly elevated and is consistent with Jag1 functioning as the biologically relevant Notch ligand in Y1 cells. Y1 cells also express both the Notch1 and Notch2 receptors together with the active signaling molecule NICD. Immunocytochemistry (ICC) reveals ubiquitous expression of core Notch pathway components (ligand: Jagged1, receptors: Notch1/2, target gene: Hes1) in Y1 cells (**Fig 2.4C**), suggesting that juxtaposed Y1 cells are capable of activating canonical Notch signaling in adjacent cells and/or are capable of self-activation (ie: ligand-mediated receptor activation).

The active engagement of Notch signaling in Y1 cells provides an appropriate model system to examine Jag1-dependent Notch activation in ACC. Because Mg2+ is required for Notch receptor stability, Mg2+ depletion can be used to induce Notch receptor cleavage and biochemically release the active NICD peptide in order to test induction of Notch-dependent transcription (as

opposed to ligand-independent constitutively active Notch-mediated transcription) in Jag1-expressing tumor cells [41, 42]. Treatment of Y1 cells for 6 hours with the chelator EDTA to deplete Mg2+ resulted in an increase in NICD protein in EDTA treated cells versus vehicle treated cells (Top panel, **Fig 2.4E**). H295A human ACC cells also exhibited a similar response to EDTA treatment (**Fig 2.4F**). To confirm NICD cleavage results in productive Notch-dependent transduction, Y1 cells were transiently transfected with a specific Notch luciferase reporter containing four NICD-consensus binding sites or an otherwise identical reporter in which the NICD sites are mutated (**Fig 2.4D**). Following EDTA treatment, an increase in the expression of the Notch luciferase reporter was observed (EDTA-Notch versus Vehicle-Notch = 2.3 fold increase, bottom panel, **Fig 2.4E**). These data indicate canonical Notch signaling can be activated in Y1 cells, presumably due to the presence of Jag1.

Because Notch signaling is generally dependent upon the juxtaposition of two adjacent cells expressing membrane-bound ligand and receptor respectively, we hypothesized that activation of Notch signaling in Y1 cells is density dependent. When Y1 cells were plated at increasing density (10%, 25%, 50%, 90% confluence) a density-dependent increase in NICD protein was observed (Top panel, **Fig 2.4G**). When the transcriptional activity of the Notch reporter was evaluated by luciferase assay, an elevated activity was observed in the highest (90%) when compared to the lowest (10%) density (High Density-Notch versus Low Density-Notch = 2.3 fold, bottom panel, **Fig 2.4G**). Additionally, immunoblot analysis did not detect Jag1 in conditioned medium from Y1 cells, which

eliminates the possibility that Jag1 is acting as a secreted factor (data not shown). Taken together, these data confirm active Notch signaling is occurring in Y1 cells.

To investigate the dependence of Notch activation on Jag1 in ACC cells, a shRNA knockdown strategy was employed. Two shRNAs with 100% homology to Jag1 mRNA were found to be sufficient to knockdown Jag1 in Y1 cells when used in combination. Two stable cell lines were generated, a Jag1-knockdown line expressing both Jag1 shRNAs [Jag1KD (GFP+)] and a control line expressing a non-specific shRNA [Scramble (GFP+)]. GFP is expressed concurrently with the shRNA and scramble vectors; thus both cell lines are GFP+. Jag1 protein was significantly decreased in the Jag1KD (GFP+) cell line, an effect that was stable for more than three weeks as determined by immunoblot analysis (**Fig 2.5A**). Jag1 mRNA expression was also reduced by 63% while the related ligand Jag2 showed no statistically significant change (**Fig 2.5B**). The concurrent suppression of the Notch target gene Hes1 is consistent with a Jag1-dependent activation of Notch signaling in ACC cells.

Jag1 has a Non-cell-autonomous Effect on ACC Cell Proliferation

To analyze the effect of Jag1 knockdown on proliferation in Y1 cells, the MTS viability assay was utilized. While no difference in proliferation was observed between Scramble (GFP+) and Jag1KD (GFP+) cells when plated at low density (10% confluence, **Fig 2.5C**), Jag1KD (GFP+) cells showed a 32% reduction in proliferation when plated at a higher concentration (40% confluence,

Fig 2.5D). These data confirm that Jag1 exerts an effect on ACC cell proliferation in a density-dependent manner. Furthermore, immunoblot analysis revealed a reduction in Proliferating Cell Nuclear Antigen (PCNA), a marker of proliferation, in the Jag1KD (GFP+) cell lysates compared to Scramble (GFP+), while Cleaved-Caspase-3 (Clv-Csp-3), a marker of apoptosis, was not detected in either cell line (**Figure 2.9A** and data not shown). These data confirm that a loss of Jag1 protein inhibits the proliferation of Y1 cells. Predicated on the assumption that Jag1 acts through Notch receptors on adjacent cells, it would be expected to influence proliferation in a non-cell-autonomous manner consistent with the density dependence observed.

In light of recent data that suggest additional Notch receptor-independent biological functions of Jag1 and the related Notch ligands DLL1 and DLL3 within the cells in which they are expressed (cell-autonomous) [25, 43, 44], a co-culture system was designed utilizing FACS analysis to further interrogate the hypothesis that Jag1 acts in a non-cell-autonomous manner in ACC cells. Jag1KD (GFP+) or Scramble (GFP+) Y1 cell lines were grown in combination with wildtype Y1 cells expressing dsRedII [wildtype Y1 (Red+)] (**Fig 2.6A**). Wildtype Y1 (Red+) cells were cultured with Scramble (GFP+) or Jag1KD (GFP+) in two different ratios: 90% Red+ / 10% GFP+ or 10% Red+ / 90% GFP+ (**Fig 2.6B**). The former condition (90% Red+ / 10% GFP+) assessed the ability of Jag1KD (GFP+) [relative to the Scramble (GFP+)] cells to proliferate upon receiving Jag1 inputs from wildtype Y1 (Red+) while the latter condition (10% Red+ / 90% GFP+) interrogates the effect of decreased Jag1 input to wildtype Y1

(Red+) cells. To guarantee sufficient cellular interactions, cells were initially plated at high density (70% confluence by Day 2, **Fig 2.6B**). To assure all cells could be analyzed at the same time at the end of the four day time course, the same initial concentration of cells were plated four days prior to analysis for Day 4, three days prior to analysis for Day 3, etc (**Fig 2.6A**). Cell number for each FACS-sorted Red+ and GFP+ populations was determined at each time point and relative proliferation of the populations is presented as a percentage change in these cell numbers from Day 1 (**Fig 2.6A**).

To examine the hypothesis that Jag1 functions in a non-cell-autonomous manner, the following mixing experiment was performed. In the 90% Red+ / 10% GFP+ ratio, Jag1KD (GFP+) cells were cultured with an abundance of wildtype Y1 (Red+) cells expressing high amounts of Jag1, thus Jag1KD (GFP+) cells should be able to receive an abundance of Jag1 signaling inputs from neighboring wildtype Y1 (Red+) cells (Left panel, **Fig 2.6B**). Under these conditions, wildtype Y1 (Red+) cells show no relative change in proliferation whether cultured with Scramble (GFP+) and Jag1KD (GFP+) cells. This is expected since wildtype Y1 (Red+) cells are most likely receiving Jag1 inputs predominantly from other wildtype Y1 (Red+) cells (Top panel, **Fig 2.6C**, **Fig 2.7**). Importantly, no difference in the proliferation of Scramble (GFP+) and Jag1KD (GFP+) cells are observed under these co-culture conditions (Bottom panel, **Fig 2.6C**, **Fig 2.7**). These data indicate that the Scramble (GFP+) and Jag1KD (GFP+) cells are capable of receiving Jag1 inputs from wildtype Y1 (Red+) cells

and, hence, proliferate normally supporting the hypothesis that Jag1 has a non-cell-autonomous effect (**Fig 2.7**).

To further test the hypothesis that Jag1 acts in a non-cell-autonomous manner on adjacent cells, wildtype Y1 (Red+) cells were cultured with Jag1KD (GFP+) or Scramble (GFP+) cells at the ratio of 10% Red+ / 90% GFP+ (Right panel, **Fig 2.6B**). Under these conditions, wildtype Y1 (Red+) cells receive a majority of signaling input from GFP+ (Scramble or Jag1KD) cells. Specifically, the wildtype Y1 (Red+) cells receive numerous Jag1 inputs from Scramble (GFP+) cells and reduced Jag1 inputs from the Jag1KD (GFP+) cells (Right panel, **Fig 2.6B**, **Fig 2.7**). Under these conditions, wildtype Y1 (Red+) cells show a 23% reduction in proliferation at Day 2 and a 27% reduction in proliferation at Day 3 (when co-cultured with Jag1KD (GFP+) cells (Top panel, **Fig 2.6D**). The Jag1KD (GFP+) cells also exhibit a maximal 35% reduction at Day 4 (Bottom panel, **Fig 2.6D**) consistent with the assumption that they are receiving the majority of signaling inputs from neighboring Jag1KD (GFP+) cells.

In summary, wildtype Y1 (Red+) cells proliferate less well when co-cultured with 90% Jag1KD (GFP+) cells suggesting that a decrease of Jag1 inputs results in retarded Y1 cell growth. Jag1KD (GFP+) cells remain competent to receive Jag1 inputs from wildtype Y1 (Red+) cells as reflected in the increased proliferation of the Jag1KD (GFP+) cells grown in the presence of 90% wildtype Y1 (Red+) cells. Jag1KD (GFP+) cells proliferated less well in the co-culture containing 90% Jag1KD (GFP+) cells suggesting that the decrease in Jag1 inputs results in retarded Jag1KD (GFP+) cell growth. Together, the co-culture

studies indicate that Jag1 enhances ACC cell proliferation in a non-cell-autonomous manner. See experimental model in **Fig 2.7**.

Inhibition of Notch-Dependent Transcription Reduces ACC Cell Proliferation

The non-cell autonomous enhancement of ACC cell proliferation by Jag1 is consistent with a Notch receptor-dependent process. As such, an inhibition of Notch-dependent transcription should phenocopy the Jag1 knockdown in a cell-autonomous manner. Notch-dependent transcription is initiated by a ternary complex of the basally-repressive CSL, active signaling molecule NICD, and transcriptional coactivator MAML(1-4) [13, 14]. An engineered peptide sequence derived from Maml1, which has a dominant-negative effect on all Notch-dependent transcription by competing for the endogenous Maml proteins and preventing their binding to NICD and CSL [29, 30] was utilized [DNMaml (GFP+): expresses GFP fusion protein of amino acids 13-74 of Maml1, Control (GFP+): expresses GFP]. Stable cell lines expressing either DNMaml or the Control construct were generated and RNA was isolated and analyzed by QPCR. In DNMaml (GFP+) cells, the canonical Notch target gene Hes1 and the putative target Cdkn1a are reduced by 64% and 43% respectively (**Fig 2.8A**). Two unrelated but highly expressed genes in Y1 cells, Ctnnb1 (β-catenin) and Sf1 (steroidogenic factor 1) were unaffected, consistent with a specific inhibition of Notch target genes in the DNMaml (GFP+) cell line.

Since DNMaml inhibits Notch-dependent transcription, we hypothesized that DNMaml (GFP+) cells would have a reduced ability to proliferate when compared to Control (GFP+) cells. Employing the MTS viability assay to assess proliferation, DNMaml (GFP+) cells plated at 40% confluence showed a 37% reduction in proliferation when compared to Control (GFP+) cells (**Fig 2.8B**). Furthermore, immunoblot analysis revealed a reduction in PCNA protein level in DNMaml (GFP+) cell lysates compared to Control (GFP+), while Clv-Csp-3 was undetectable and the protein level of Cleaved-Caspase-6 (Clv-Csp-6), another marker of apoptosis, was unchanged (**Fig 2.9B**).

While Jag1 functions non-cell-autonomously to influence ACC cell proliferation, DNMaml targets downstream Notch signaling and should have a cell-autonomous effect on proliferation. To directly address this supposition, a similar co-culture study was performed using a 50% wildtype Y1 (Red+) / 50% Control (GFP+) or DNMaml (GFP+) ratio. Wildtype Y1 (Red+) cells co-cultured with either Control (GFP+) or DNMaml (GFP+) cells maintain robust proliferation (Left panel, Fig 8C). No statistically significant difference in Hes1 expression was observed on Day 4 in wildtype Y1 (Red+) cells cultured with GFP+ (Control or DNMaml) cells indicating DNMaml is not affecting Notch signaling in adjacent wildtype Y1 (Red+) cells (Right panel, **Fig 2.8C**). Conversely, DNMaml (GFP+) cells cultured with wildtype Y1 (Red+) cells exhibit a 34.3% reduction in proliferation when compared to Control (GFP+) cells cultured with wildtype Y1 (Red+) cells (Left panel, **Fig 2.8D**). Hes1 mRNA was reduced 71.34% in DNMaml (GFP+) versus Control (GFP+) cells at Day 4 (Right panel, **Fig 2.8D**).

These data indicate DNMaml is acting specifically in DNMaml (GFP+) cells. Together with the Jag1 co-culture studies, these data support a Jag1 dependent activation of Notch signaling in ACC that can be targeted at the level of ligand (presenting cell) or receptor (receiving cell) to inhibit ACC cell proliferation.

Notch receptor antibodies inhibit Notch target gene expression in Y1 cells while γ-secretase*.inhibitors have no effect*

Next, we asked whether Jag1-dependent activation of Notch signaling required γ-secretase complex mediated cleavage of the intracellular domain of the Notch receptor and the resultant generation and release of the active NICD [13, 14]. γ-secretase inhibitors, such as Dapt and Compound E, have been shown to be effective in inhibiting canonical Notch signaling in numerous systems [45], such as the MOLT4 T-ALL cell line[46]. Y1 cells and MOLT4 cells (used as a positive control) were treated with either vehicle or 100μM of DAPT and harvested for protein and mRNA analysis after 24h (**Fig 2.10A, B**). As expected, a loss of NICD protein and Hes1 expression was oberserved in the MOLT4 cell line with DAPT treatment. However, we surprisingly did not see loss of NICD or reduction in Hes1 expression in DAPT-treated Y1 cells or Y1 cells treated with an additional γ-secretase inhibitor, Compound E (γ-secretase inhibitor-XXI) (**Fig 2.10C, D**). Because NICD release from the Notch receptor is required for Notch pathway activation, we then chose to directly inhibit the Notch receptors using inhibitory Notch antibodies. These antibodies have previously

been shown to stabilize the negative regulatory region (NRR) of the Notch receptors and prevent S2 cleavage, a critical step in activation of Notch receptors[14, 38]. Treatment of Y1 cells with 80ng of either Notch1 or Notch2 antibody revealed a significant decrease in Hes1 mRNA expression after 4h but not 24h (**Fig 2.10C**). Treatment with both Notch antibodies resulted in a significant decrease in Hes1 expression after 4h and 24h. These results are consistent with ligand-dependent Notch receptor cleavage and release of NICD in Y1 cells.

JAG1 expression is correlated with increased aggressiveness of ACC

JAG1 is upregulated in ACC and acts through canonical Notch signaling to enhance density-dependent ACC cell proliferation. To determine whether elevated JAG1 mRNA expression levels in human ACC correspond to an increase in cancer aggressiveness, tumor stage and grade (as assessed by mitotic rate) were examined in the 33 ACC samples used in the microarray analysis. JAG1 mRNA expression levels correlated with advanced stage ($r=0.35$; $p=0.04$) and with mitotic rate ($r=0.40$; $p=0.02$) (**Fig 2.11A, B**). Specifically, JAG1 expression was increased 1.67 fold ($p=0.05$) in late stage ACC (Stage III and IV) compared to early stage ACC (Stage I and II) (**Fig 2.11A**). Our previous microarray has shown strong correlations between KI67 and Topoisomerasse 2A (TOP2A) expression, two markers of proliferation that are highly upregulated in ACC, and immunohistochemical staining for Ki67 and Top2a protein [39]. We

identified a positive correlation of JAG1 expression with KI67 expression (overall correlation r=0.62, p<0.0001) and TOP2A (overall correlation r=0.69, p<0.0001) (**Fig 2.11C, D**). These data are consistent with the significant role of JAG1 in ACC cell proliferation and advanced stage of disease.

Discussion

The Notch ligand JAG1 mRNA and protein are upregulated in adrenocortical carcinoma (ACC). JAG1 upregulation can be modeled in the Y1 mouse ACC cell line that expresses Jag1, Notch receptors, and downstream signaling molecules. Y1 cells exhibit density-dependent Notch activation. Jag1 enhances cell proliferation through activation of canonical Notch signaling as shown through knockdown and co-culture experiments. Inhibition of Notch signaling at the level of ligand (Jag1KD) or post receptor signaling (DNMaml), results in similar inhibition of cell proliferation. Analysis of clinical data indicates Jag1 expression correlates with both Grade and Stage of ACC supporting a role of JAG1-dependent Notch activation in ACC.

JAG1 upregulation has been observed in several cancers such as breast and prostate cancer where it facilitates proliferation and metastasis [24, 47]. In breast cancer, JAG1 is correlated with poor prognosis and lower survival rates in women with late stage, aggressive cancer [20-22]. Mechanistically, JAG1 has been shown to induce expression of cylinD1 in prostate cancer [23], enhance the

number of cancer cells in S-phase [48], and facilitate proliferation in Wnt1-transformed breast epithelial cells [49, 50].

In addition to Notch ligands, Notch receptors Notch2 and Notch3 may be concomitantly upregulated in ACC. Upregulation of Notch2 receptors has been reported in several other cancers [51, 52] including gastric cancer, where an upregulation of Jag1 has also been observed [17, 53]. Notch2 does not show a positive correlation to JAG1 expression, which suggests that Notch2 and JAG1 upregulation may be independent events. In contrast, expression of one Notch3 probe set exhibits a positive correlation to JAG1 expression. Similarly, in lung cancer, JAG1 and Notch3 are upregulated in addition to enhancing proliferation of lung cancer cells, an effect that appears to be Notch3 dependent [54, 55]. However, the stoichiometry of Notch receptor-ligand binding in adrenal tumors cells is unknown. The lack of a consistent upregulation amongst all Notch receptor probe sets suggests that in order to accommodate the upregulation of JAG1 observed in late stage ACC, a concomitant upregulation of Notch receptors may not be required.

Furthermore, the Notch target gene Hey1 is upregulated in ACC and its expression is positively correlated to JAG1 expression while the related Notch target gene Hes1 does not. It is interesting that Hes1 appears to be Jag1-mediated Notch target in Y1 cells but may not in human tumor. It is possible that a unique set of Notch-regulated target genes is distinct in each of these systems. The full complement of genes that are regulated by Notch signaling in ACC is unknown and may include a plethora of non-canonical targets and/or the

expression profile of Notch-regulated genes may be distinct to each individual tumor.

While the canonical mechanism by which Jag1 mediates cellular effects in numerous systems is through its binding to the Notch receptors and activation of downstream signaling [11-14], Jag1 and the other Notch ligands may also have receptor independent roles [25, 43, 44]. Overexpression of Jag1 has been shown to cell-autonomously induce transformation of RKE cells independent of Notch receptors but dependent on intracellular interaction between the cytoplasmic tail of Jag1 and Affadin, a cell adherens junction protein [25]. Furthermore, Jag1 and DLL1 are able to be processed by the γ-secretase complex to release intracellular signaling fragments [43]. In this report, knockdown of Jag1 in mouse adrenocortical cancer cells employing specific shRNAs resulted in a density-dependent reduction in proliferation. Co-culture experiments of normal Y1 cells with either Jag1KD or Scramble cell lines tested whether Jag1 has a cell-autonomous or non-cell-autonomous effect. Jag1KD cells were competent to proliferate provided they received sufficient Jag1 signaling inputs from adjacent cells. Cells receiving diminished Jag1 inputs from Jag1KD cells did not proliferate as well as cells receiving inputs from control (Scramble) cells. These data indicate Jag1 does not have a cell-autonomous effect but instead mediates adrenal cancer cell proliferation by binding to and activating Notch receptors on adjacent cells. The similar cell-autonomous reduction of growth following inhibition of Notch-dependent transcription utilizing a dominant negative version

of the transcriptional coactivator Maml1 supports the conclusion that Jag1 effects ACC cell proliferation in a non-cell-autonomous manner.

Of obvious interest is the molecular mechanism of JAG1 upregulation in human ACC. It is informative that Wnt and Notch are known to synergize in a variety of developmental systems such as the ear where Jag1 acts to mediate some of the effects of downstream Wnt/β-Catenin signaling on the formation of the otic placode [56]. Moreover, JAG1 has been shown to be a direct target of β-catenin in the epidermis where Notch signaling is required for β-catenin mediated melanoma formation [57, 58]. A synergistic effect between Notch and Wnt on tumorigenesis is also seen in breast and colon carcinoma where JAG1 is upregulated in both of these cancers [15, 20, 49, 59].

Whether the Notch and Wnt pathways interact in ACC is unknown. While repression of Notch-dependent transcription had no effect on β-catenin (Ctnnb1) expression in the DNMaml experiments (**Fig 8A**), it remains unknown if Wnt activation synergizes or activates various components of the Notch pathway. Nuclear β-catenin has been observed in both benign ACAs and malignant ACCs[5-9] as well as the known ACC cell lines H295A. Whether JAG1 is a downstream target of Wnt signaling in ACC is currently unknown. Additionally, mouse models of ACC in which β-catenin is constitutively active have been recently reported[10, 60]. It would be informative to examine whether β-catenin activation has an effect on Jag1 and other Notch factor expression in this model. Furthermore, conditional knockout of Jag1 in Wnt/β-catenin-induced colorectal tumors results in a reduction in tumor size when compared to tumors in which

Jag1 expression is not genetically altered [61]. Understanding the mechanism of JAG1 upregulation in ACC will be an important area of investigation.

The correlation of high JAG1 levels with high grade and late stage ACC in the current study is provocative and suggests JAG1 upregulation is a later event in ACC. Activating mutations of β-catenin have been identified in both ACA and ACC which suggests activation of the Wnt pathway may be a crucial step in initiation of adrenocortical tumorigenesis [5-10, 60]. Furthermore, the insulin-like growth factor (IGF) pathway ligand, IGF2, has been shown to be the most differentially upregulated gene in ACC [26, 62]. Taken together, these data suggest the Wnt activation concurrent with upregulation of IGF2 may be critical molecular events required for ACC development. Recently, our group has shown that a mouse model in which Wnt and IGF pathways are overactivated develops adrenal tumors that manifest as histopathologically more advanced than tumors in mice where only Wnt or IGF pathways are perturbed[60]. These data support the hypothesis that Wnt and IGF pathways synergize to drive adrenocortical tumor growth. However, the lack of penetrance of ACC formation in these mice argues against the IGF and Wnt pathways as sufficient to induce progression to *bona fide* ACC. The correlation of JAG1 expression with late stage ACC suggests its upregulation may be a molecular event that antecedes that activation of Wnt and IGF pathways. Targeting Jag1-mediated Notch signaling may be a potential novel target for therapy, possibly in tandem with inhibition of IGF and/or Wnt signaling.

Figure 2.1: JAG1 is upregulated in human ACC. A. Heatmap of Affymetrix U133A 2.0 Plus oligonucleotide array representing Notch ligand genes. Normal Adrenal (NL), n=10, Adrenocortical Adenoma (ACA) n=22, Adrenocortical Carcinoma (ACC), n=33). Scale is indicated. **B.** Dot plot of two JAG1 and JAG2 probe sets. Each dot indicates one tissue sample. Lines indicate mean expression levels. JAG1 #1: ACC vs NL * $p=4x10^{-6}$, ACC vs ACA # $p=7x10^{-12}$), JAG1 #2: ACC vs NL** $p=1x10^{-6}$, ACA vs NL ## $p=2x10^{-11}$), JAG2 #1: ACC vs NL *** $p=6x10^{-4}$, ACC vs ACA ### $p=4x10^{-4}$), JAG2 #2: ACC vs NL **** $p=5.3x10^{-3}$, ACC vs ACA #### $p=3x10^{-4}$). **C.** QPCR analysis of mRNA from randomly selected human samples (NL: n=5, ACA: n=5, ACC: n=10) for JAG1 and JAG2. Each data point represents an average of triplicate determinations. **D.** Correlation of Log-transformed JAG1 QPCR expression data (from Fig 1C) with the corresponding JAG1 (probe set #2) microarray data (from Fig 1B) (r=0.874, $p=5x10^{-7}$) and JAG2 QPCR data with JAG2 (probe set #1) microarray data (r=0.545, p=0.013)**. E.** Top panel: Immunoblot analysis of 5µg of protein lysates from randomly selected human adrenal samples (NL: n=5, ACA: n=5, ACC: n=10). Blots were probed for Jagged1 and β-actin used as a loading control. Molecular weights are indicated. Bottom panel: Quantification of Immunoblots. Jag1 protein was normalized to β-Actin and then to NL sample #06. The average of two experiments is presented. Line represent mean.

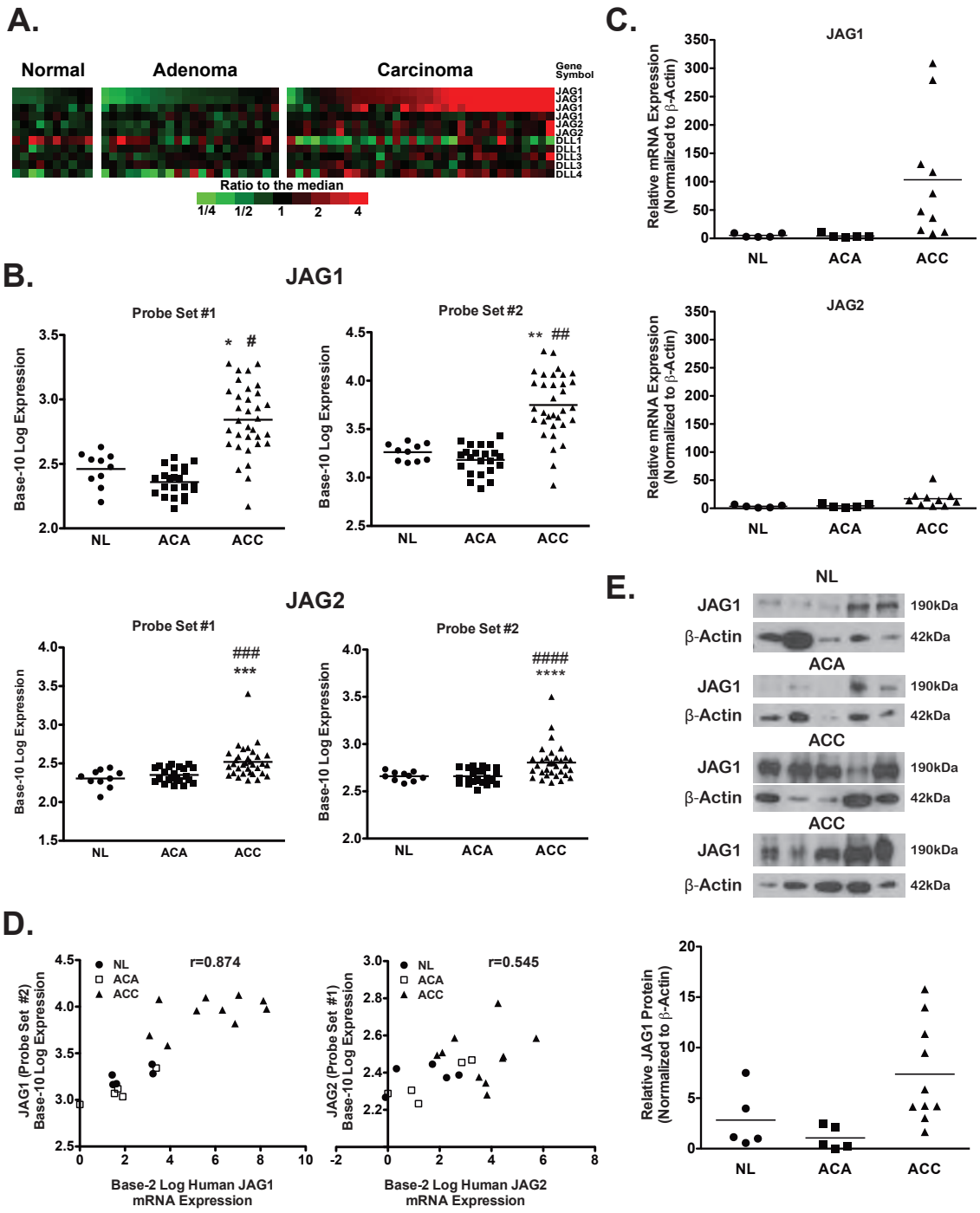

A.
Normal
Adenoma
Carcinoma
Gene Symbol
JAG1
JAG1
JAG1
JAG2
JAG2
DLL1
DLL1
DLL3
DLL3
DLL4
Ratio to the median
1/4 1/2 1 2 4
B.
JAG1
Probe Set #1
Probe Set #2
Base-10 Log Expression
NL ACA ACC
JAG2
Probe Set #1
Probe Set #2
Base-10 Log Expression
NL ACA ACC
C.
JAG1
Relative mRNA Expression (Normalized to β-Actin)
NL ACA ACC
JAG2
Relative mRNA Expression (Normalized to β-Actin)
NL ACA ACC
D.
JAG1 (Probe Set #2) Base-10 Log Expression
NL
ACA
ACC
r=0.874
Base-2 Log Human JAG1 mRNA Expression
JAG2 (Probe Set #1) Base-10 Log Expression
NL
ACA
ACC
r=0.545
Base-2 Log Human JAG2 mRNA Expression
E.
NL
JAG1
β-Actin
190kDa
42kDa
ACA
JAG1
β-Actin
190kDa
42kDa
ACC
JAG1
β-Actin
190kDa
42kDa
ACC
JAG1
β-Actin
190kDa
42kDa
Relative JAG1 Protein (Normalized to β-Actin)
NL ACA ACC

Figure 2.2: Notch receptors exhibit variable expression in human ACC. A. Dot plot of two Notch1, Notch2 and Notch3 and one Notch4 probe sets. Each dot indicates one tissue sample. Lines indicate mean expression levels. Normal Adrenal (NL), n=10, Adrenocortical Adenoma (ACA) n=22, Adrenocortical Carcinoma (ACC), n=33. Notch2 #1: * ACC vs ACA p = 0.01, # ACA vs NL p=0.003. Notch3 #2: ** ACC vs ACA p=0.008, ACC vs NL ## p=0.01. **B.** Correlation of JAG1 (Probe Set #2) microarray expression with Notch2 (Probe Set #1), overall correlation r=0.056, p=0.656, and Notch3 (Probe Set #2), overall correlation r=0.29, p=0.021. **C.** QPCR analysis of mRNA from randomly selected human samples (NL: n=5, ACA: n=5, ACC: n=10) Notch2. Each data point represents an average of triplicate determinations.

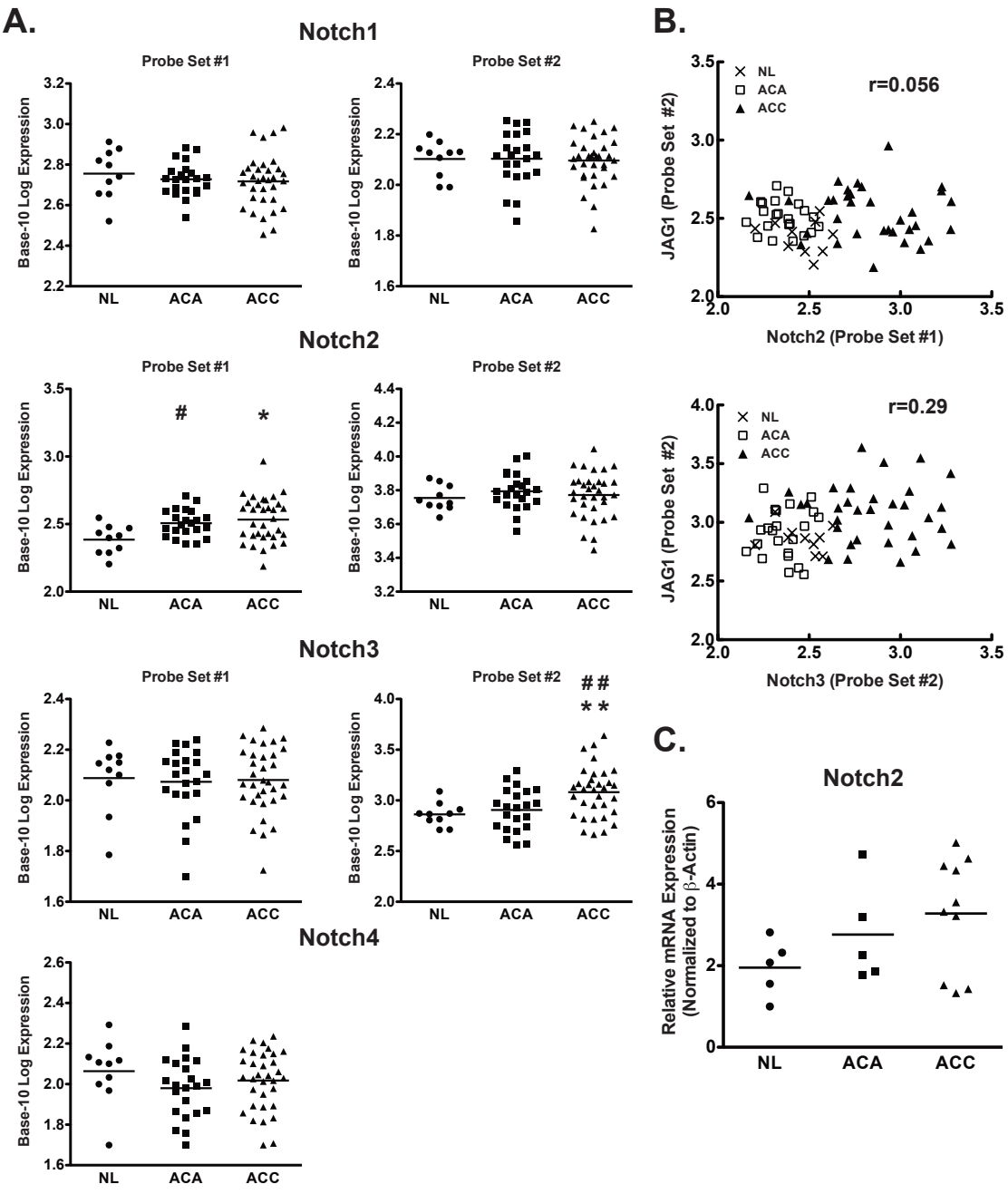

A.
Notch1
Probe Set #1
Probe Set #2
Base-10 Log Expression
NL
ACA
ACC
Notch2
Probe Set #1
Probe Set #2
#
*
Notch3
Probe Set #1
Probe Set #2
# #
* *
Notch4
B.
NL
ACA
ACC
r=0.056
JAG1 (Probe Set #2)
Notch2 (Probe Set #1)
r=0.29
Notch3 (Probe Set #2)
C.
Notch2
Relative mRNA Expression
(Normalized to β-Actin)

Figure 2.3: Notch target genes are upregulated in human ACC. A. Dot plot of two Hes1 and Hey1 probe sets. Each dot indicates one tissue sample. Lines indicate mean expression levels. Normal Adrenal (NL), n=10, Adrenocortical Adenoma (ACA) n=22, Adrenocortical Carcinoma (ACC), n=33. Hey1 #1: * ACC vs ACA p = 0.0004, # ACC vs NL p=0.0007. Hey1 #2: ** ACC vs ACA, p=0.0008, ACC vs NL ## p=0.005. **B.** Correlation of JAG1 (Probe Set #2) microarray expression with Hey1 (Probe Set #1), overall correlation r=0.34, p=0.0053, and Hey1 (Probe Set #2), overall correlation r=0.34, p=0.0054. **C.** QPCR analysis of mRNA from randomly selected human samples (NL: n=5, ACA: n=5, ACC: n=10) for Hes1 and Hey1. Each data point represents an average of triplicate determinations.

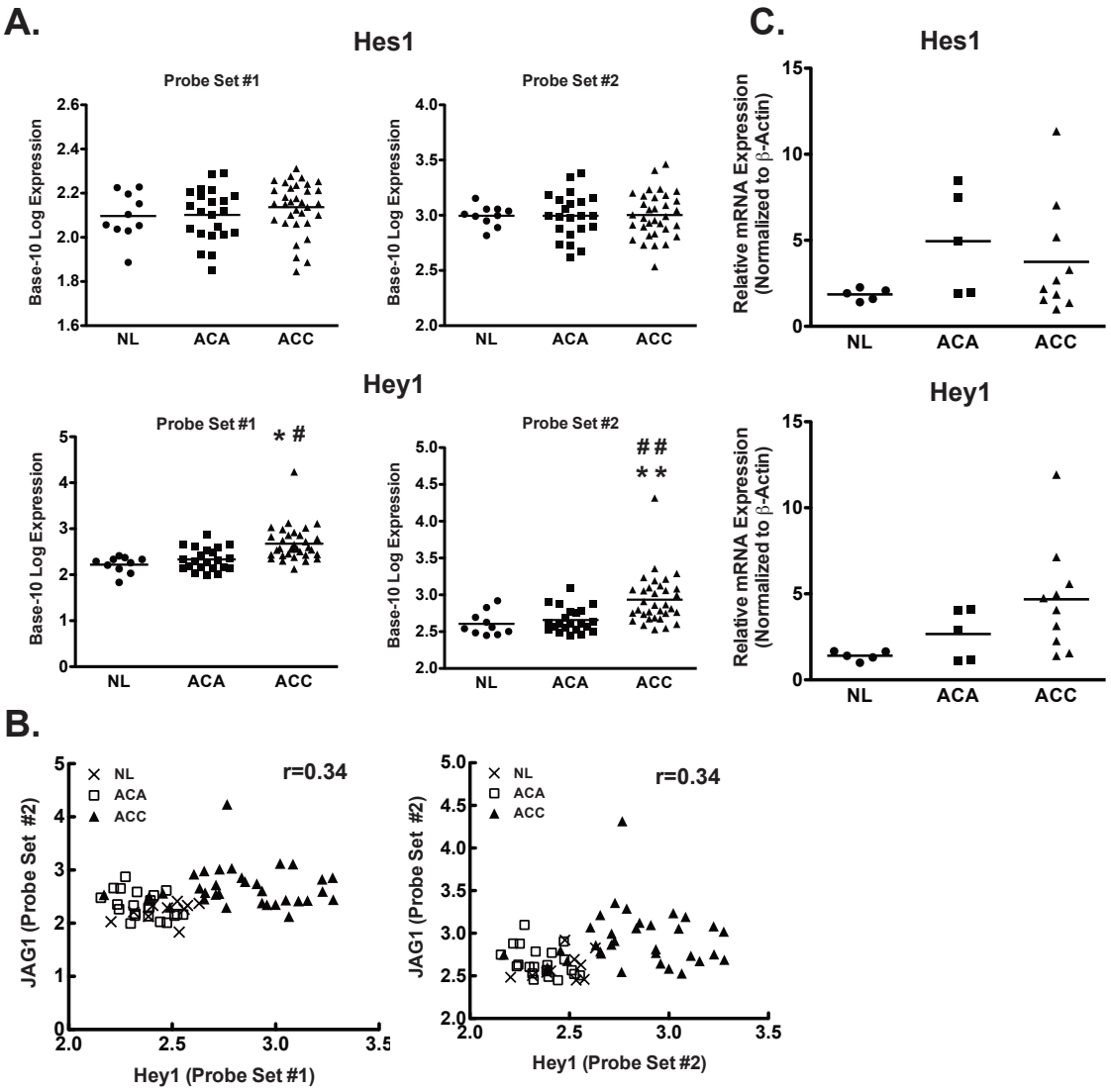

A.
Hes1
Probe Set #1
Probe Set #2
Base-10 Log Expression
NL
ACA
ACC
Hey1
* #
* *
C.
Relative mRNA Expression (Normalized to β-Actin)
B.
r=0.34
JAG1 (Probe Set #2)
Hey1 (Probe Set #1)
Hey1 (Probe Set #2)

Figure 2.4: The Y1 mouse ACC cell line exhibits active Notch signaling. A. Immunoblot analysis of 10µg of protein lysate from WT mouse adrenal (Adrenal), mouse ACC cell line (Y1), and human ACC (H295, RL251) cell lines for Jag1, Notch1, Notch2, and NICD. β-actin is used as loading control. **B***.* Efficiency-corrected ΔCT QPCR method of triplicate samples used to quantify the expression of the 5 Notch ligands in Y1 cells (* JAG1 vs JAG2, p=0.00001). Representative experiment of three repetitions. **C.** Immunofluorescent colocalization of Jag1 with Notch1, Notch2, and Hes1 in Y1 cells. **D.** Luciferase constructs for the Notch reporter, which contains 4 CSL binding sites, and Control reporter, which the 4 CSL sites are mutated. **E.** Top panel: Immunoblot analysis of 10µg of protein lysate from Y1 cells treated with 5mM EDTA or vehicle (PBS) for 6h. Blots were probe for NICD and β-actin, used as loading control. Bottom panel: Luciferase assay of triplicate samples of Notch (4xwtCBF1Luc; pJH23A) and Control (4xmtCBF1Luc; pJH25A) reporter in Y1 cells treated with 5mM EDTA or vehicle for 6h. Luciferase expression is normalized to p-RL-TK *Renilla* expression (EDTA, Notch: * vs EDTA, Control p=0.004, # vs Vehicle, Notch p=0.0039). Representative experiment of three repetitions. **F.** Immunoblot analysis of 10µg of protein lysate from H295A cells treated with 2.5mM and 5mM EDTA or vehicle (PBS) for 6h. Blots were probe for NICD and β-actin, used as loading control. **G.** Top panel: Immunoblot analysis of NICD levels of 10µg of protein lysates of Y1 cells grown at 10%, 25%, 50%, 90% confluence. β-actin is used as loading control. Bottom panel: Luciferase assay of triplicate samples of Notch and Control reporter expression (normalized to

Renilla) in Y1 cells grown at 10% (low density) and 90% (high density) confluence (High density, Notch: * vs High density, Control p=0.00003, # vs Low density, Notch p=0.0006). Representative experiment of three repetitions.

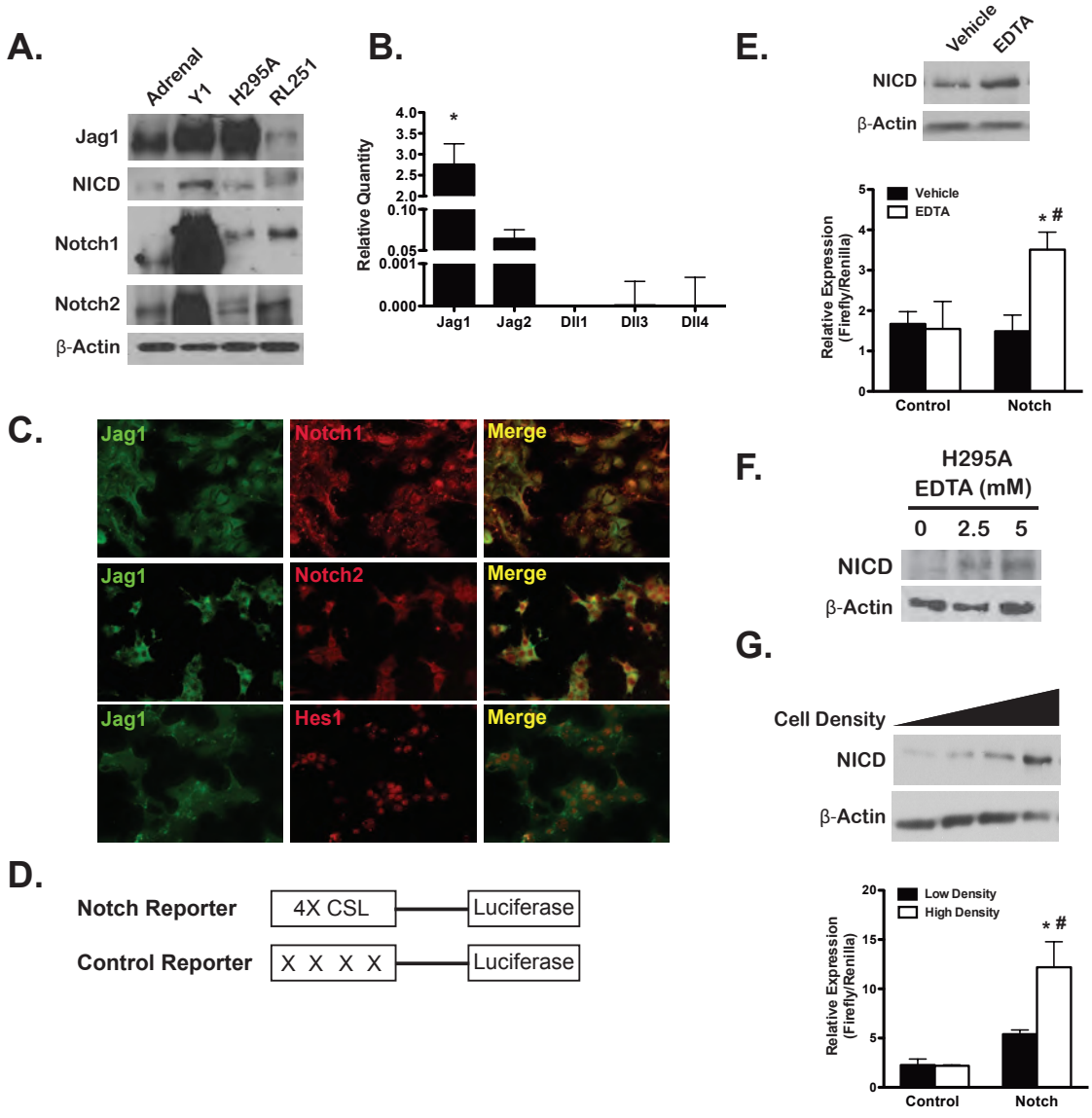


Figure 2.5: Jag1 knockdown in Y1 cells inhibits proliferation in a density-dependent manner. A. Immunoblot analysis of protein lysates from stable cell lines expressing shRNAs for either Scramble or Jag1 [Scramble (GFP+) and Jag1KD (GFP+) respectively]. Blots were probed for Jag1 and β-actin, used as loading control. **B.** QPCR analysis of mRNA from Scramble (GFP+) and Jag1KD (GFP+) stable cell lines analyzed by the ΔΔCT method, and normalized to β-actin [Scramble (GFP+) vs Jag1KD (GFP+): * Jag1 p=0.0002, # Hes1 p=0.001]. Representative experiment of five repetitions. Absorbance values obtained from MTS viability assay on cell lines [Scramble (GFP+) vs Jag1KD (GFP+)] plated at (**C.***)* 10% confluence at Day 1 and growth to 35% confluence by Day 4 and (**D.**) 40% confluence at Day1 and growth to 85% by Day 4, Scramble (GFP+) vs Jag1KD (GFP+) at Day 4, * p=0.0045. Each data point represents an average ± SD of 6 determinations. Representative experiment of four repetitions.

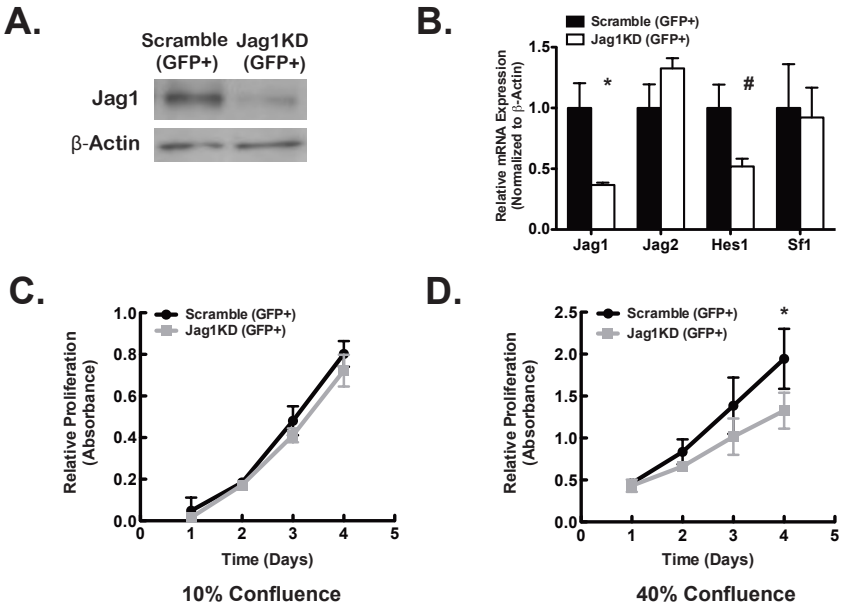
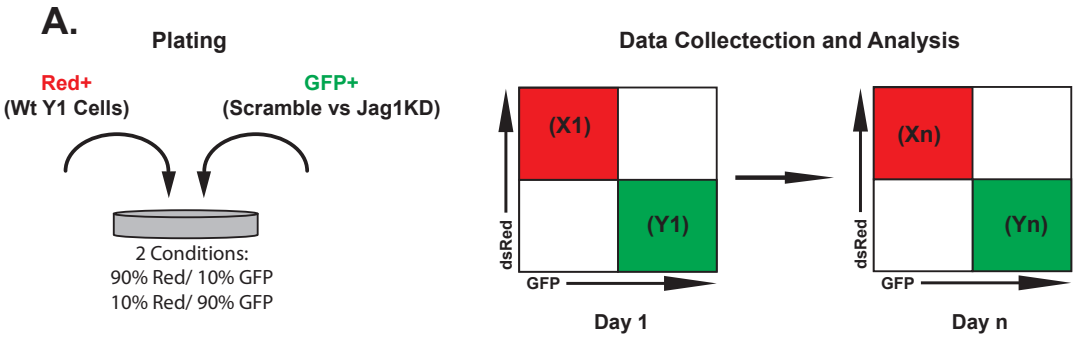

A.
Scramble (GFP+)
Jag1KD (GFP+)
Jag1
β-Actin
B.
Scramble (GFP+)
Jag1KD (GFP+)
Relative mRNA Expression (Normalized to β-Actin)
1.5
1.0
0.5
0.0
*
#
Jag1
Jag2
Hes1
Sf1
C.
Scramble (GFP+)
Jag1KD (GFP+)
Relative Proliferation (Absorbance)
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Time (Days)
10% Confluence
D.
Scramble (GFP+)
Jag1KD (GFP+)
*
Relative Proliferation (Absorbance)
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Time (Days)
40% Confluence

Figure 2.6: **Jag1 enhances ACC proliferation in a non-cell-autonomous manner. A.** Schematic indicating experimental design. dsRedII+ (normal Y1) cells were co-cultured with GFP+ (Scramble or Jag1KD) cells in ratios 90% Red+/ 10% GFP+, or 10% Red+/ 90% GFP+. Initial combined cell number (Red+ plus GFP+) was 150,000 cells and triplicate wells were plated. The same initial plating was used for each time point and cells were plated 4 days from harvest for the Day 4 timepoint, 3 Days from the harvest for the Day 3 timepoint, etc. Harvested cells were analyzed by FACS. 10,000 sorted cells were counted for each timepoint and the number of Red+ and GFP+ determined for each count. The percentage change in cell number from Day 1 was determined by the formulas indicated and based on the 10,000 cells counted for each time point. **B.** Immunocytochemical images of the two different co-culture conditions at Day 2. **C.** Top panel: The percentage change of Red+ cells from Day 1 for each timepoint in the 90% Red+/10% GFP+ condition. Bottom panel: The percentage change of GFP+ cells from Day 1 for each timepoint in the 90% Red+/10% GFP+ condition. **D.** Top panel: The percentage change of Red+ cells from Day 1 for each timepoint in the 10% Red+/90% GFP+ condition (* $p<0.03$). Bottom panel: The percentage change of GFP+ cells from Day 1 for each timepoint in the 10% Red+/90% GFP+ condition. Scramble (GFP+) vs Jag1KD (GFP+) at Day 2, 3, and 4, * $p<0.0001$). Each bar represents an average ± SD of 3 determinations. Representative experiment of three repetitions.



Identical Plating for 4 Day Timecourse

Data analyzed by the formulas (Xn/X1) ×100 and (Yn/Y1) × 100 for each day n where X = Red+ population and Y = GFP+ population

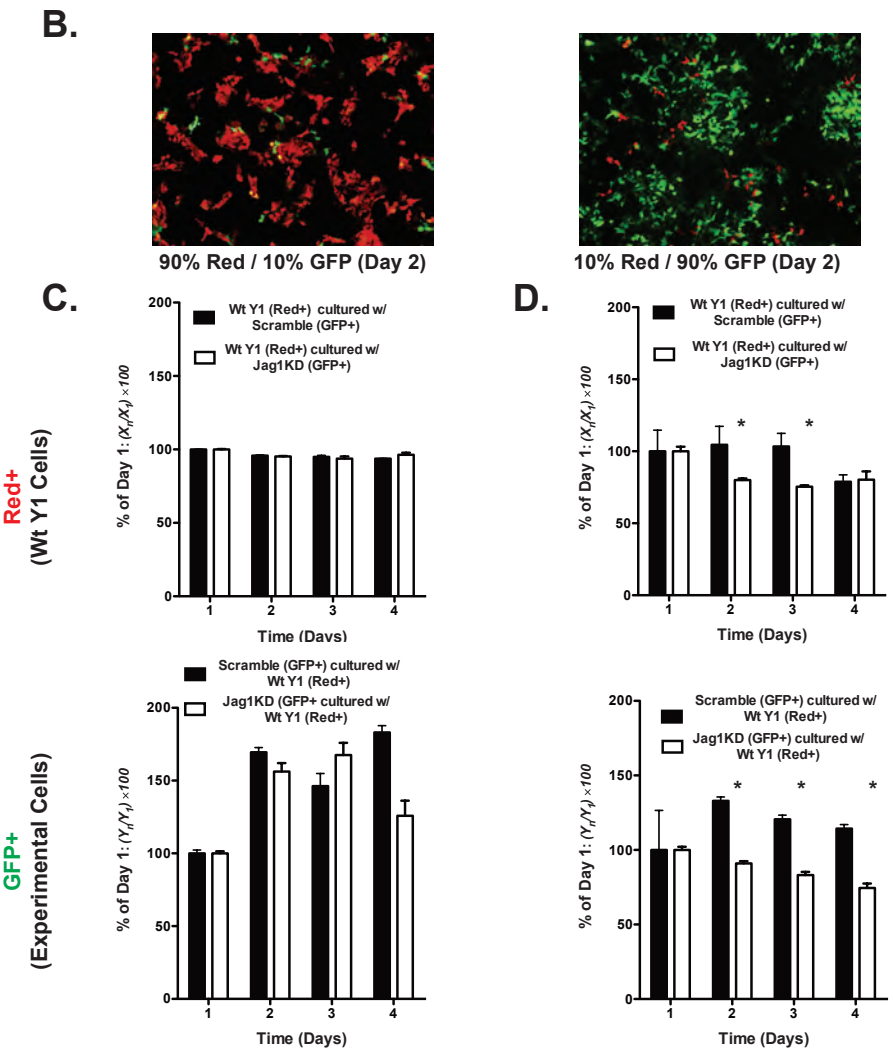


Figure 2.7: A model of Jag1-Notch signaling occurring in co-culture experiments. A visual representation of the types of signaling occurring between normal Y1 (Red+) and Scramble/Jag1KD (GFP+) cells in the two co-culture conditions. Jag1KD cells (Right panel) are able to receive Notch inputs (90/10) but not send them (10/90). Red+ and Scramble cells (Left panel) are both able to signal through Jag1 and receive signals from Jag1.

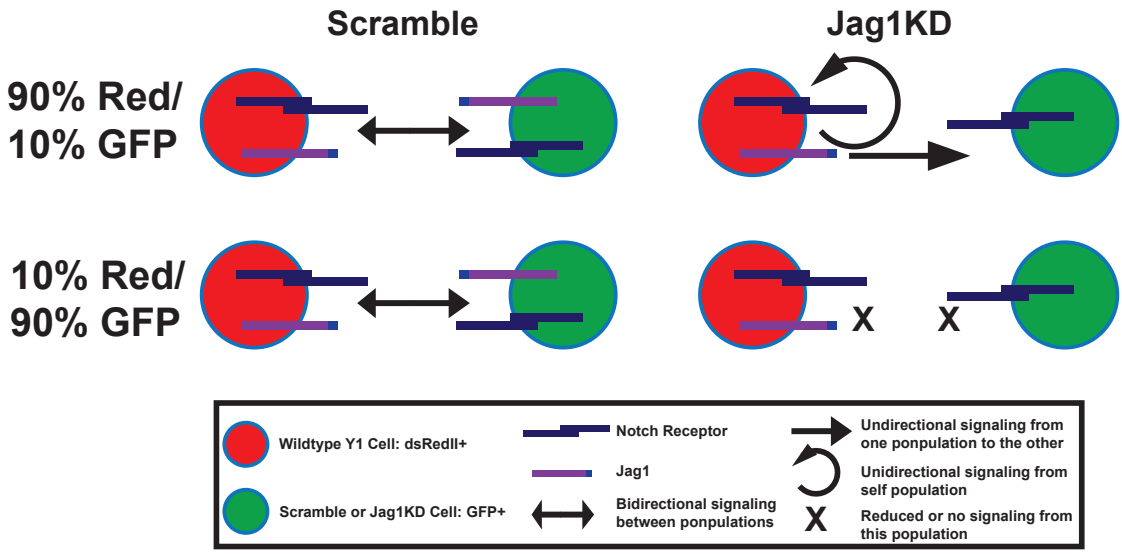

Scramble
Jag1KD
90% Red/
10% GFP
10% Red/
90% GFP
X
X
Wildtype Y1 Cell: dsRedII+
Scramble or Jag1KD Cell: GFP+
Notch Receptor
Jag1
Bidirectional signaling between ponpulations
Undirectional signaling from one ponpulation to the other
Unidirectional signaling from self population
X
Reduced or no signaling from this population

Figure 2.8: DNMaml suppression of Notch-dependent transcription reduces Y1 cell proliferation to a similar degree as Jag1 Knockdown. A. QPCR of mRNA from stable cells lines expressing either Control (GFP+) or DNMaml (GFP+) constructs analyzed using the ΔΔCT method and normalized to β-actin [Control (GFP=) vs DNMaml (GFP+): Hes1 * p=0.0001, Cdkn1a # p=0.02]. **B.** Absorbance values obtained from MTS viability assay on cell lines [Control (GFP+) vs DNMaml (GFP+), * p<0.0001]. Each data point represents an average ± SD of 6 determinations. Representative experiment of four repetitions. Co-culture of 50% normal Y1 cells (Red+) and either 50% Control (GFP+) or 50% DNMaml cells (GFP+). Initial combined cell number (Red+ plus GFP+) was 150,000 cells and triplicate wells were plated. The same initial plating was used for each time point and cells were plated 4 days from harvest for the Day 4 timepoint, 3 Days from the harvest for the Day 3 timepoint, etc. Harvested cells were analyzed by FACS. 10,000 cells were counted for each timepoint and the number of Red+ and GFP+ determined for each count. The percentage change in cell number from Day 1 was determined by the formulas indicated (y-axis) and based on the 10,000 cells counted for each time point, **C.** Left panel: The percentage change of Red+ cells from Day 1 for each timepoint. Right panel: mRNA was harvested from Red+ cells at the Day 4 timepoint for the Control and DNMaml co-culture. Hes1 expression was determined by the ΔΔCT method and normalized to β-actin. β-catenin. **D.** Left panel: The percentage change of GFP+ cells from Day 1 for each timepoint [Control (GFP+) vs DNMaml (GFP+), * p<0.0001, # p=0.06]. Right panel: mRNA was harvested from GFP+ cells at the

Day 4 timepoint for the Control and DNMaml co-culture. Hes1 expression was determined by the ΔΔCT method and normalized to β-actin (* p=0.0066). Each bar represents an average ± SD of 3 determinations. Representative experiment of three repetitions.

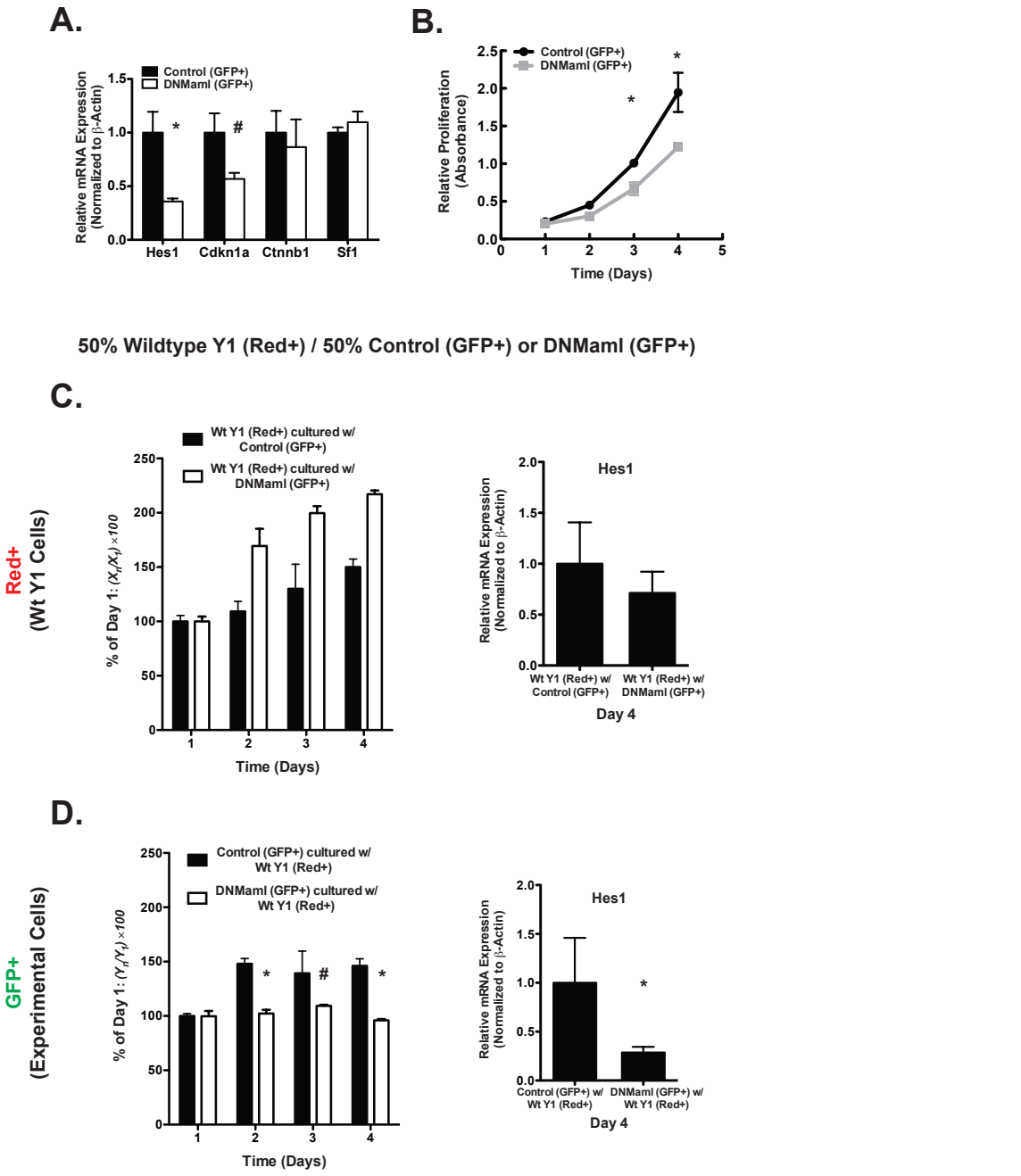


Figure 2.9: **Jag1 knockdown or inhibition of Notch-dependent transcription in Y1 cells reduces proliferation but has no effect on apoptosis. A.** Immunoblot analysis of protein lysates from stable cell lines expressing shRNAs for either Scramble or Jag1 [Scramble (GFP+) and Jag1KD (GFP+) respectively]. Blots were probed for Jag1, Proliferating Cell Nuclear Antigen (PCNA) and β-actin, used as loading control. **B.** Immunoblot analysis of protein lysates rom stable cells lines expressing either Control (GFP+) or DNMaml (GFP+) constructs. Blots were probed for PCNA, Cleaved-Caspase-6 (Clv-Casp-6) and β-actin, used as loading control.

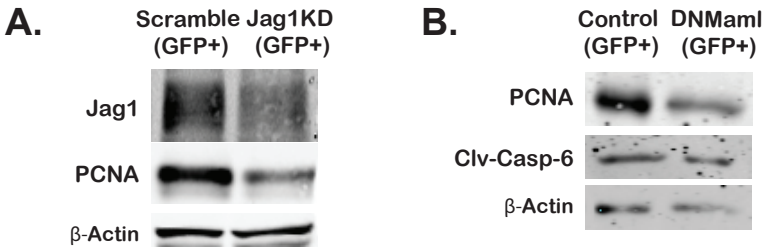

A.
Scramble (GFP+)
Jag1KD (GFP+)
Jag1
PCNA
β-Actin
B.
Control (GFP+)
DNMaml (GFP+)
PCNA
Clv-Casp-6
β-Actin

Figure 2.10: Notch receptor antibodies inhibit Notch target gene expression in Y1 cells. A. Immunoblot analysis of 15µg of protein lysate from MOLT4 and Y1 cells treated with vehicle (DMSO) or 100µM, 500µM DAPT for 24h. Blots were probed for NICD and β-actin, used as loading control. **B.** QPCR analysis for Hes1 of mRNA in triplicate from vehicle or 100µM treated Y1 and MOLT4 cells analyzed by the ΔΔCT method, and normalized to β -actin. Representative of triplicate experiments. MOLT4, Vehicle vs 100µM DAPT, * $p < 0.0001$ **C.** Immunoblot analysis of 15µg of protein lysate from Y1 cells treated with 100, 250, 400, and 500ng of Compound E or vehicle (DMSO) for 24h. Blots were probed for NICD and β-actin, used as loading control. **D.** QPCR analysis for Hes1 of mRNA in triplicate from vehicle or 100, 250, 400, and 500ng Compound E treated Y1 cells analyzed by the ΔΔCT method, and normalized to β -actin. Representative of triplicate experiments. **E.** QPR analysis of Hes1 mRNA in triplicate from vehicle, 80ng anit-Notch1 antibody, anti-Notch2 antibody, or combination of both antibodies after 4h and 24h treatments. Average of duplicate experiments. Notch1Ab, 4h or Notch2Ab, 4h vs Vehicle, 4h * $p < 0.002$, Notch1/2Ab, 4h vs Vehicle, 4h # $p < 0.001$, Notch1/2Ab, 24h vs Vehicle, 24h, # $p < 0.001$.

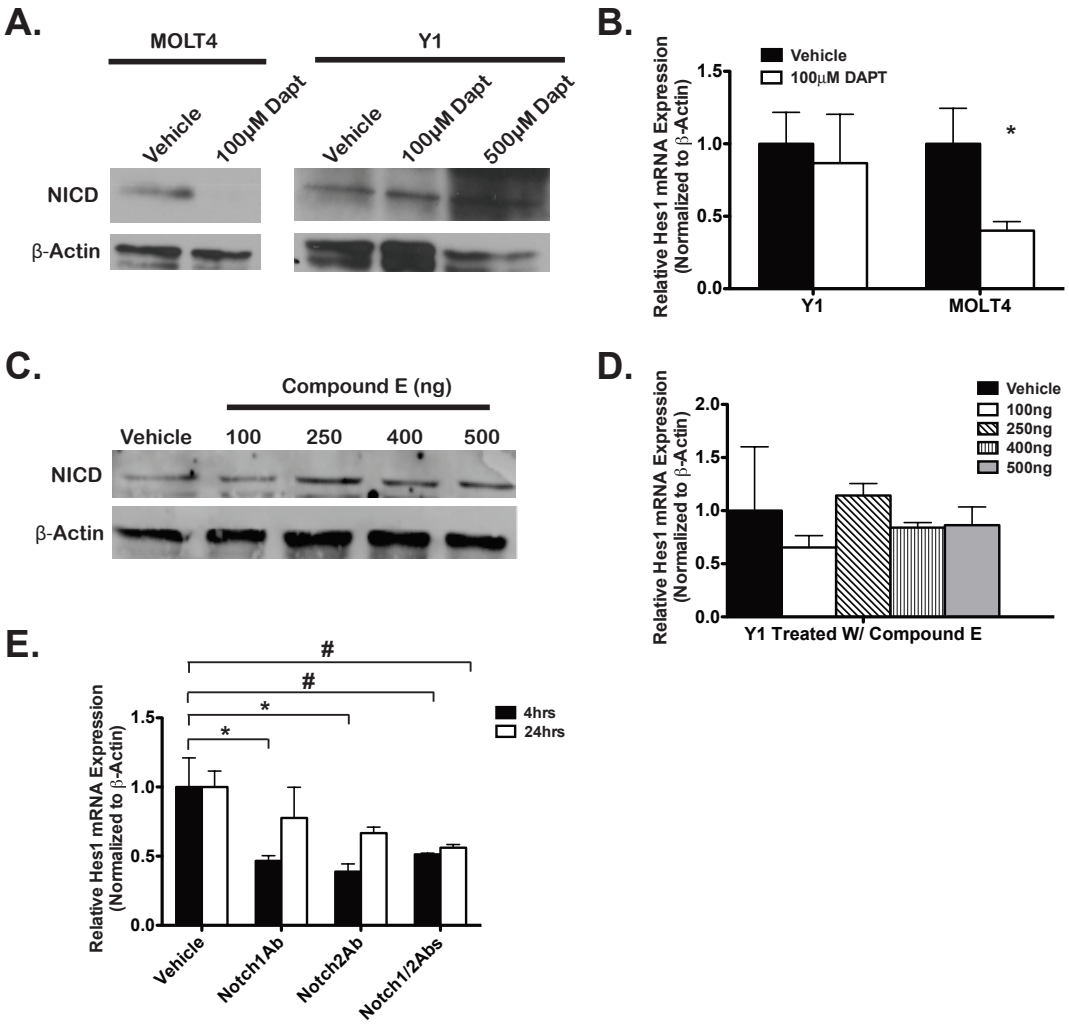

A.
MOLT4
Y1
Vehicle
100μM Dapt
Vehicle
100μM Dapt
500μM Dapt
NICD
β-Actin
B.
Vehicle
100μM DAPT
Relative Hes1 mRNA Expression (Normalized to β-Actin)
1.5
1.0
0.5
0.0
*
Y1
MOLT4
C.
Compound E (ng)
Vehicle
100
250
400
500
NICD
β-Actin
D.
Vehicle
100ng
250ng
400ng
500ng
Relative Hes1 mRNA Expression (Normalized to β-Actin)
2.0
1.5
1.0
0.5
0.0
Y1 Treated W/ Compound E
E.
#
#
*
*
4hrs
24hrs
Relative Hes1 mRNA Expression (Normalized to β-Actin)
1.5
1.0
0.5
0.0
Vehicle
Notch1Ab
Notch2Ab
Notch1/2Abs

Figure 2.11: JAG1 expression is highest in aggressive, highly proliferating ACC. A. Correlation of JAG1 expression (base-2 log transformed) for stage in ACCs (n=33). 19 Stage I + II vs 14 Stage III + IV p=0.0551, overall correlation r=0.35, p=0.04. **B.** Correlation of JAG1 expression (base-2 log transformed) with mitotic rate (base-2 log transformed). Overall correlation r=0.40, p=0.02. **C.** Correlation of JAG1 expression (base-2 log transformed) with KI67 expression (base-2 log transformed) across all human adrenal samples used in the microarray data set. Overall correlation r = 0.62, p<0.0001. **D.** Correlation of JAG1 expression (base-2 log transformed) with TOP2A expression (base-2 log transformed) across all human adrenal samples used in the microarray data set. Overall correlation r=0.69, p<0.0001.

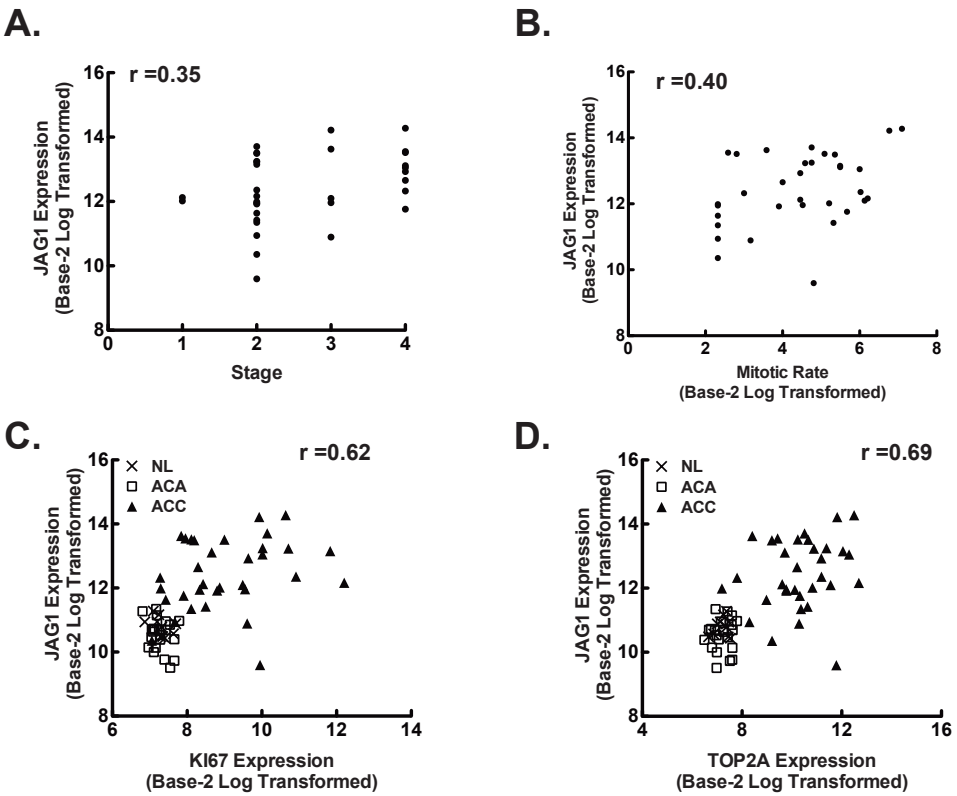

A.
r =0.35
JAG1 Expression
(Base-2 Log Transformed)
Stage
B.
r =0.40
JAG1 Expression
(Base-2 Log Transformed)
Mitotic Rate
(Base-2 Log Transformed)
C.
r =0.62
NL
ACA
ACC
JAG1 Expression
(Base-2 Log Transformed)
KI67 Expression
(Base-2 Log Transformed)
D.
r =0.69
NL
ACA
ACC
JAG1 Expression
(Base-2 Log Transformed)
TOP2A Expression
(Base-2 Log Transformed)

Table 2.1: Notch gene microarray probe sets

Probe Set ID	Gene Name	Probe Set #
231183_s_at	JAG1	1
209099_x_at	JAG1	2
209784_s_at	JAG2	1
32137_at	JAG2	2
2188902_at	NOTCH1	1
223508_at	NOTCH1	2
155743_at	NOTCH2	1
202442_x_at	NOTCH2	2
203237_s_at	NOTCH3	1
203328_s_at	NOTCH3	2
240786_at	NOTCH4	1
203393_at	HES1	1
203395_s_at	HES1	2
218839_at	HEY1	1
44783_at	HEY1	2

Table 2.2: QPCR primers

Primer Name	Species	Forward Sequence	Reverse Sequence
JAG1	Human	5'-tgccaagtgccaggaagt-3'	5'-gccccatctggtatcacact-3'
JAG2	Human	5'-tgggactgggacaacgatac-3'	5'-atgcgacactcgctcgat-3'
Beta-Actin	Human	5'-tgacaggatcgagaaggaga-3'	5'-cgctcaggaggagcaatg-3'
JAG1	Mouse	5'-gaggcgtcctctgaaaaaca-3'	5'-acccaagccactgttaagaca-3'
JAG2	Mouse	5'-tcctcctgctgctttgtgat-3'	5'-tgtcaggcaggtcccttg-3'
DLL1	Mouse	5'-acagaggggagaagatgtgc-3'	5'-ccctggcagacagattgg-3'
DLL3	Mouse	5'-tcgtacgtgtgcccttcc-3'	5'-tgctctctccaggtttcaatg-3'
DLL4	Mouse	5'-aggtgccacttcggttacac-3'	5'-gggagagcaaatggctgata-3'
HES1	Mouse	5'-acaccggacaaaccaaagac-3'	5'-cgcctcttctccatgatagg-3'
CDKN1A	Mouse	5'-tccacagcgatatccagaca-3'	5'-ggacatcaccaggattggac-3'
SF1	Mouse	5'-tccagtacggcaaggaagac-3'	5'-ctgtgctcagctccacctc-3'
CTNNB1	Mouse	5'-gcagcagcagtttgtgga-3'	5'-tgtggagagctccagtacacc-3'
Beta-Actin	Mouse	5'-ctaaggccaaccgtgaaag-3'	5'-accagaggcatacagggaca-3'

Table 3: Antibodies and uses.

Antibody Name	Species	Use	Company	Catalog #
Jagged1 (C-20)	Goat	IB, ICC, OD	Santa Cruz	Sc-6011
Hes1	Rabbit	ICC	Millipore	AB5702
Cleaved Notch1 (Val1744)	Rabbit	IB	Cell Signaling	2421
Notch1 (C-20)	Goat	ICC	Santa Cruz	Sc-6014
Notch2 (25-255)	Rabbit	ICC	Santa Cruz	Sc-5545
btan20 (Notch1)	Rat	IB	Iowa DHSB	na
C651.6DbHN (Notch2)	Rat	IB	Iowa DHSB	na
Beta-Actin	Mouse	IB	Sigma	A-5411
Anti-Goat IRDye 800 CW	Donkey	OD	LICOR	926-32214
Anti-Mouse IRDye 680	Goat	OD	LICOR	926-32220
Anti-Goat IgG HRP	Rabbit	IB	Thermo Scientific	31433
Anti-Mouse IgG HRP	Goat	IB	Pierce	31434
Anti-Rabbit IgG HRP	Goat	IB	Pierce	31462
Anti-Rat HRP	Goat	IB	Pierce	31475
Anti-Rabbit Dylight 549	Goat	ICC	Jackson ImmunoResearch	111-506-045
Anti-Goat Dylight 549	Rabbit	ICC	Jackson ImmunoResearch	305-505-045
Anti-Goat Dylight 488	Rabbit	ICC	Jackson ImmunoResearch	305-485-045

IB: Standard Immunoblot
OD: Immunoblot using Odyssey IR Scanner
ICC: Immunocytochemistry

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Chapter 3: The Role of Notch signaling in Normal Adrenal Biology

Abstract

The adrenal gland is an endocrine organ composed of the embryologically and functionally distinct cortex, which secretes steroid hormones, and medulla, which secretes catecholamine hormones. Notch signaling is a highly conserved developmental signaling pathway that has been shown to be involved in development and/or maintenance of numerous organ systems. Notch signaling has been implicated in the development of sympathetic ganglia, which are of the same embryonic lineage of the adrenal medulla, but Notch has never been considered in chromaffin cell development and/or function. We have identified Notch ligands, receptors, and canonical target genes Hes1 and Hes5 are expressed in the adrenal medulla. Inhibition of canonical Notch signaling in TH+ cells, a marker of all chromaffin cells, results in an upregulation of expression of the catecholamine biosynthetic enzyme TH while overactivation of Notch signaling in TH+ cells results in a reduction in expression of the epinephrine-synthesizing enzyme PNMT. Furthermore, inhibition of canonical Notch signaling in Sf1+ cells had no effect on the function or histology of the adrenal cortex, but did manifest with abnormalities in the ovary. These data suggest Notch signaling

may be important in modulating the production of catecholamine hormones from the adrenal medulla and may have implications for pheochromocytoma, a tumor derived from chromaffin cells of the adrenal medulla.

Introduction

The adrenal glands are endocrine organs located superior to the kidneys that are responsible for the coordination of the mammalian stress response. The mesoderm-derived cortex secretes the glucocorticoids cortisol and corticosterone while the neuroectoderm-derived medulla secretes catecholamine hormones [1]. The adrenal medulla is primarily involved in mitigation of the “fight or flight” through secretion of the catecholamine hormones Epinephrine and to a lesser extent, Norepinephrine [2].

Chromaffin cells of the adrenal medulla are derived from the sympathadrenal (SA) lineage, a population of progenitors cells originating from the neural crest (NC) that give rise to sympathetic ganglia, the adrenal medulla, and extra-adrenal paraganglia (e.g “the organ of Zuckerkandl”) [3]. Many studies have elucidated the role of a host of transcription factors in SA specification and chromaffin cell development, such as Mash1 [4], Phox2B [5], Phox2A [6], dHand [7], Gata3 [8], and Sox protein[9] (See review [10]. Signaling pathways such as the bone morphogenic protein (BMP) pathway have also been identified as crucial for determination of SA fate and migration [11, 12]. Furthermore, glucocorticoid signaling from the adrenal cortex to the adrenal medulla has been

implicated in the maturation of epinephrine-secreting (adrenergic) chromaffin cells through induction of the epinephrine-synthesizing enzyme phenylethanolamin-N-methyltansferase (PNMT) [13-15]. However, the role of signaling pathways mitigating migration of chromaffin progenitors into the adrenal primordia and specifying chromaffin cell fate during development in the post-migratory adrenal environment have not been considered.

The Notch signaling pathway is an evolutionarily conserve pathway that has been implicated in the development and/or maintenance of numerous organ systems [16-18]. Canonical Notch signaling occurs between interaction between transembrane ligands and receptors expressed on adjacent cells that culminates in the release of the active signaling molecule, the cleaved Notch intracellular domain. In the avian embryo, Notch pathway members are expressed in the neural ectoderm adjacent the neural tube and have been implicated in specification of the NC lineage [19, 20]. Furthermore, Notch signaling may suppress differentiation of sympathetic neurons, which suggest a selective role of Notch signaling in SA development [21]. However, the function of the Notch pathway in chromaffin cell development has not been considered.

We report that active Notch signaling occurs in chromaffin progenitors of the fetal medulla and in mature chromaffin cells of the adult medulla. Tissue-specific inhibition of canonical Notch signaling in TH+ cells results in an upregulation of TH mRNA and proteins. Furthermore, overactivation of Notch signaling in TH+ cells reduces the expression of PNMT in chromaffin cells. As shown through inhibition of Notch signaling in Sf1+ cells, Notch signaling appears

to be dispensable for the overall development and maintenance of the adrenal cortex. These data provide evidence for a putative role of Notch signaling in modulating chromaffin cell expression of catecholamine synthesizing enzymes.

Materials and Methods

Mouse lines

All mouse lines including *Hes1*$^{+/GFP}$ and *Hes5*$^{+/GFP}$[22], *TH*$^{+/Cre}$[23], *Sf1*$^{+/Cre}$,[24] *R26R-Tom*$^{+/EGFP}$[25], *R26R*$^{+/DNMaml}$[26], and *R26R*$^{+/NICD}$[27] have been previously characterized. TH$^{+/Cre}$ and R26R$^{+/NICD}$ were obtained from the Jackson Laboratory (Bar Harbor, ME). Hes1$^{+/GFP}$ and Hes5$^{+/GFP}$ lines were generously provided by Dr. Linda Samuelson (University of Michigan, Ann Arbor, MI). R26R$^{+/DNMaml}$ was generously provided by Dr. Ivan Maillard (University of Michigan, Ann Arbor, MI). 26R-Tom$^{+/EGFP}$ was generously provided by Dr. Andrzej Dlugosz (University of Michigan, Ann Arbor, MI). Some lines were bred to homozygosity as indicated. For timed breedings, embryos were staged by designating noon of the day in which the copulatory plug was detected as E0.5. Genders of embryos was determined with SRY genotyping of the yolk sac. Lines were maintained on mixed genetic backgrounds. For a complete list of genotyping primers, see **Table 3.1**. All animal protocols were approved by the University of Michigan Institutional Animal Care and Use Committee.

Immunohistochemical and immunofluorescent analysis

Adrenals were harvested and fixed in 4% paraformaldehyde for 1.5-4hrs (depending on the age of the animals and the type of cross employed) at 4°C. Tissues were dehydrated in graded ethanol solutions (50% and 70%) followed by paraffin embedding. 6μm sections were cut using a microtome. Sections were sequentially rehydrated by two 5min incubations in each xylenes, 95% and 100% ethanol, and water. For histological analysis, slides incubated directly in hematoxylin and eosin (H&E) solutions for 2min each and then dehydrated in the opposite manner as described above. Paramount was used to coverslip the slides.

For immunofluorescent stains, dehydration of tissue sections was performed as described above. Antigen retrieval was performed by boiling rehydrated sections in 10mM (pH6) or 5mM (pH2) sodium citrate buffer for 10min followed by 20min cooling step. Sections were washed 1X in phosphate-buffered saline (PBS) for 2min then blocked with in 3% milk in PBS solution for 4-5hrs at 4°C (or 1hr at room temperature). Slides were washed 1X in PBS then incubated in primary antibodies diluted in 0.3% milk in PBS overnight at 4°C. For a complete list of primary and secondary antibodies used in this chapter, see **Table 3.3**. Slides were then washed three times for 5min in PBS and then incubated in immunofluorescnt secondary antibodies diluted in 0.3% milk in PBS for 2hrs at room temperature. Slides were then washed one time in PBS then counterstaned with Dapi (1:1000) for 5min. Three additional 5min PBS washes were performed. Cover slips were applied and images obtained as previously

described[28, 29]. For some sections, detection of primary antibodies was performed with a chromogenic approach using diaminobenzidine (Sigma, St. Louis, MO).

Quantitative real time-PCR (QPCR) analysis

Adrenals were homogenized and then RNA isolated with the RNAeasy mini kit (Qiagen, Valencia, CA). cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad laboratories, Hercules, CA). QPCR experiments were performed as previously described[28, 29]. A comprehensive list of QPCR primers can be found in **Table 3.2**. Analysis was conducted with either the efficiency-corrected Δ CT method of the $\Delta\Delta$ CT method as indicated[30]. Expression of mRNA was normalized to β-actin. Statistical analyses of QPCR experiments were made using student's T-test.

Immunoblot analysis

Analysis of protein lysates from homogenized adrenal glands was performed as previously described[28]. Analysis of protein lysate was conducted as described but blocking was done in Odyssey Blocking buffer (Licor, Lincoln, NE), secondary antibodies were used were odyssey IRdyes (**Table 3**). Quantifications were performed using the Odyssey software.

Results

Canonical Notch target genes Hes1 and Hes5 are expressed in the adrenal medulla.

Notch signaling has previously been reported in sympathetic ganglia, which are of the same developmental lineage as chromaffin cells [21]. In order to identify whether active Notch signaling was also occurring in chromaffin cells of the adrenal medulla, we employed a transgenic reporter mouse approach. Hes1 and Hes5 are two bHLH genes that have been shown to be direct targets of canonical Notch signaling in a variety of systems [16]. Two transgenic mice in which EGFP expression is driven by a 2.5kb region of the Hes1 promoter (*Hes1*$^{+/GFP}$) or by a 0.76kb region of the Hes5 promoter (*Hes5*$^{+/GFP}$) have previously been reported (**Fig 3.1A**, See **Fig 3.1** for a description of all mouse lines employed in this chapter) [22]. Both lines of Hes transgenic mice were bred to homozygosity in order to maximize detection of the GFP signal (*Hes1*$^{GFP/GFP}$ and *Hes5*$^{GFP/GFP}$, respectively).

Immunofluorescent detection of GFP protein revealed Hes1 promoter activity throughout the adrenal medulla at birth (P0) and throughout adult life up to 23wks of age (later time points were not examined) (**Fig 3.2A**). Tyrosine Hydroxylase (TH), the first enzyme in catecholamine biosynthesis that is expressed in every chromaffin cell of the adrenal medulla, and Steroidogenic Factor 1 (Sf1), a transcription factor required for steroidogensis that is expressed in every adrenocortical cell, were used to delineate the adrenal medulla and

cortex respectively (**Fig 3.2A, B**) [3, 24, 31]. Hes1 expression was detected exclusively in the adrenal medulla as indicated by costains with TH (**Fig 3.2A**, top row). While a vast majority of TH+ cells appeared to be GFP+, some GFP- cells were detected suggesting Hes1 is not expressed at the same level in every chromaffin cell.

Hes5 expression was also detected in the adrenal medulla as indicated by immunofluroescent detection of GFP protein in *Hes5*$^{GFP/GFP}$ transgenic mice. In contrast to Hes1, in neonatal mice (P5), Hes5 expression was not detected throughout the adrenal medulla but was localized to discrete clusters of TH+ cells (**Fig 3.2B**, top row). In adult mice at 4wks of age, similar cell clusters were detected but they seemed to localize towards the periphery of the medulla (**Fig 3.2B**, bottom row).

Notch signaling has been implicated in the formation of the neural crest but its role in SA progenitor cells during development has not been delineated [20, 32]. To investigate the role of Notch signaling in chromaffin progenitors of the SA lineage, we performed timed breedings using *Hes1*$^{GFP/GFP}$ and *Hes5*$^{GFP/GFP}$ mice (**Fig 3.3**). We examined embryos for Hes1 expression at E12.5, the approximate time point at which chromaffin progenitors begin to invade the adrenal primordial [1, 33]. As expected, robust TH expression was detected ventral to the dorsal aorta (DO), where SA progenitors are believed to mature (**Fig 3.3A**, top row). GFP protein was also detected throughout the same region but appeared to exhibit a broader expression pattern. However, the adrenal primordial was not detected in these sections.

We examined animals in which we can detect TH+ cells migrating into the adrenal primordial, which are presumed to be chromaffin cell progenitors (**Fig 3.3A**, bottom two rows) [10]. Many of the TH+ cells appeared to concurrently express GFP but an additional population of GFP+ cells lacked TH expression. These GFP+/TH- cells seemed to reside within the adrenal primordial itself but since GFP costaining with Sf1 was not performed, the identity of this population could not be confirmed. Chromaffin progenitors have previously been observed to invade the adrenal primordia from the dorsal side (the "head" of the adrenal primordia) [34]. When examined at higher magnification, the population of TH+ cells that was oriented dorsal of the adrenal primordial was almost entirely GFP+, suggesting that chromaffin progenitors engage in active Notch signaling prior to invasion of the adrenal anlage (**Fig 3.3A**, bottom two rows, far right panels, 400X magnification).

Next, we considered the activity of Notch signaling in post-migratory environment of the developing fetal adrenal gland. In E14.5 *Hes5*$^{GFP/GFP}$ mice, we detected Hes5+ (GFP+) cells throughout the adrenal medulla and many of these cells colocalized with TH (**Fig 3.3B**). Similar to the expression pattern observed in neonatal mice, Hes5 expression was not detected in every TH+ cell. In contrast, in E16.5 *Hes1*$^{GFP/GFP}$ mice, GFP expression was detected throughout the adrenal medulla, presumably in every TH+ cell, but this was not confirmed since costaining with TH was not performed at this time point (**Fig 3.3C**). Taken together, these data indicate that canonical Notch target genes Hes1 and Hes5

are expressed in TH+ chromaffin cells of the adult and developing adrenal medulla, which suggests that active Notch signaling is occurring in these cells.

Notch pathway ligand and receptors are expressed in the adrenal medulla

The expression of Hes1 and Hes5 strongly indicates that active Notch signaling is occurring in the medulla but since Hes1 transcription can be initiated through other pathways [35, 36], we wondered if Notch ligands and receptors are concomitantly expressed in the adrenal medulla. First, we performed relative expression QPCR using the efficiency-correct ΔCT method to determine the relative quantities of the five canonical Notch ligands (Jag1/2, Dll1/3/4) in male and female mice between P0 and 50wks of age (**Fig 3.4A**) [37]. At every time point for both genders, Jag1 was the predominant ligand present while Jag2 and Dll4 were also robustly detected but were present in lower quantities than Jag1. Dll1 and Dll3 were expressed at considerably lower levels compared to the other three ligands. Next, using the same QPCR data but analyzed using the ΔΔCT method, we determined that change in expression of the Notch ligands over time in both male and female mice (**Fig 3.4B**). Despite variation at some of the time points, the expression of Notch ligands was constant throughout adult mouse life.

To confirm the QPCR data, immunofluorescent detection of Dll4 (**Fig 3.4C)** and Jag1 (**Fig 3.4D)** was performed**.** Because the expression profile of Notch ligands was virtually identical between males and females, we decided to only consider male mice. Dll4 was detected in discrete clusters of TH+ cells

throughout the adrenal medulla at 3, 23 and 50wks of age (**Fig 3.4C**). In contrast, Jag1 expression was detected throughout the medulla. The broader expression pattern of Jag1 compared to Dll4 may account for the increased quantity of Jag1 compared to Dll4. Interestingly, Jag1 was also detected in the subcapsule of the adrenal cortex, a region believed to contain adrenal progenitor cells (**Fig 3.4D,** bottom panel) [38, 39]. These data prove that Notch ligands are expressed in the adrenal medulla concurrent with Hes1 and Hes5 expression.

In addition to the five canonical ligands, the identification of a host of non-canonical ligands has been reported (for review, see [37]). One of the most studied is the Dlk1, a transmembrane protein that shares a remarkable degree of structural similarity to the canonical Notch ligands [40]. Expression of Dlk1 has previously been identified in chromaffin progenitors pre- and post-migration into the adrenal primordia, as well as chromaffin cells of the adult medulla [41, 42]. As expected, immunofluorescent detection of Dlk1 was detected throughout the adrenal medulla (**Fig 3.5**). Dlk1 expression was detected in every TH+ cell of the fetal medulla at E14.5, when migration of chromaffin progenitors into the fetal adrenal is complete, (**Fig 3.5A)** and in the postnatal medulla (**Fig 3.5B**). Dlk1 expression was maintained throughout adult life (**Fig 3.5C**). These data confirm the previous reports that Dlk1 is expressed in fetal and adult chromaffin cells. Therefore, in addition to the canonical ligands Jag1 and Dll4, Dlk1 may be involved in activation and/or modulation of Notch activity in the adrenal medulla.

The presence of the Notch ligands in medulla provides another lines of evidence that active Notch signaling is occurring in chromaffin cells. We

extended our analysis to the four Notch receptors that have been identified in mice (**Fig 3.6**) [16, 17]. Using the same mRNA samples and analysis as described in **Fig 3.4**, we analyzed the relative quantities of the Notch receptors in male and female mice between 0 and 50wks of age (**Fig 3.6A**). At early time point in both genders, Notch2 was the predominant ligand expressed while at later time points, Notch1 appeared to be more robustly expressed than Notch2 in male mice. Notch1 and Notch2 were expressed at comparable quantities in later time points in female mice, concurrent with increased detection of Notch4. At every time point in both genders, Notch3 expression was lower than the other three receptors.

As with the analysis of the ligands, the QPCR data were reanalyzed to examine changes in expression of the Notch receptors over time (**Fig 3.6B**). In male mice, Notch2-4 expression remained constant over time while Notch1 expression increased, which confirmed the greater relative quantities of Notch1 detected in the previous analysis. In female mice, expression of Notch1-3 remained constant over time while an upregulation of Notch4 was identified, which confirmed the increased quantities of Notch4 detected in later time points in the previous analysis. Despite the increased in Notch4 expression in older mice, Notch1 and Notch2 appear to be the predominant receptors expressed in both male and female mice, and we therefore focused our analysis on these two receptors.

To confirm the QPCR data, immunofluroescent detection of Notch1 and Notch2 was performed in male mice (**Fig 3.6C, D**). Both Notch1 and Notch2

exhibited a broad expression pattern throughout the adrenal medulla. Notch2 expression colocalized with TH confirming that Notch2 is expressed in TH+ chromaffin cells of the medulla. However, the expression of Notch2 was not the same in every TH+ cells (**Fig 3.6C**, left panel). Several TH+, Notch2- cells were detected at higher magnification, which suggests that activation of Notch2-dependent signaling is not the same in all chromaffin cells (**Fig 3.6C**, bottom panel). In contrast, Notch1 expression appeared similar in most cells, although the intensity of the Notch1 signal seemed greater at the periphery of the adrenal medulla. These data taken together with the Notch ligand and target gene expression studies, confirm that active Notch signaling is occurring in chromaffin cells of the adrenal medulla. However, the specific type of Notch-ligand interactions occurring between chromaffin cells cannot be precisely determined from these studies.

Inhibition of canonical Notch signaling in TH+ chromaffin cells results in an upregulation of TH expression

We have shown that Notch receptors, ligands, and canonical target genes are expressed in the TH+ chromaffin cells of the adrenal medulla. In order to interrogate the function of Notch in these cells, we took advantage of Cre-Lox technology to inhibit canonical Notch signaling in TH+ cells. This was accomplished using the $TH^{+/Cre}$ line in which Cre recombinase is knocked into the 3' UTR of the endogenous *TH* gene [23] (**Fig 3.1B**). This mouse has previously

been reported to target 100% of TH+ cells of the adrenal medulla [23]. In order to test the recombination efficiency of this line, we crossed the *TH*$^{+/Cre}$ line to the *R26R-Tom*$^{+/EGFP}$ line (**Fig 3.7**). The *R26R-Tom*$^{+/EGFP}$ line is a dual fluorescent reporter mouse that expresses the Tomato fluorescent protein in the absence of Cre, but in the presence of Cre, the Tomato gene is excised and EGFP is expressed instead (**Fig 3.1C**) [25]. Therefore, TH+ cells that express Cre recombinase should also express EGFP. However, as determined by immunofluroescent detection of GFP protein, only a portion of adrenal medullary cells in *TH*$^{+/Cre}$:*R26R-Tom*$^{+/EGFP}$ mice expressed GFP (**Fig 3.7**, right panels). As a control, no GFP was detected in *TH*$^{+/+}$:*R26R-Tom*$^{+/EGFP}$ mice (**Fig 3.7**, left panels). Two different *TH*$^{+/Cre}$ mice were crossed to two different *R26R-Tom*$^{+/EGFP}$ mice but the result was the same for both *TH*$^{+/Cre}$ lines. The lack of complete recombination in every TH+ cell conflicts with the previously reported expression in the adrenal medulla of this mouse [23].

Despite the mosaic expression pattern of the Cre, we used the *TH*$^{+/Cre}$ line to inhibit canonical Notch signaling in TH+ cells by crossing it to the *R26R*$^{+/DNMaml}$ line. *R26R*$^{+/DNMaml}$ is a *Rosa26* locus knock-in mouse that employs a NeoSTOP cassette upstream of the DNMaml-EGFP fusion protein, a peptide derived from amino acids 13-74 of Maml1 that has been shown to inhibit all downstream canonical Notch signaling [26, 43] (**Fig 3.1D**). In the presence of Cre recombinase expression, the NeoSTOP cassette is excised and the DNMaml peptide is expressed [26].

We analyzed the *TH*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ mice through immunofluorscent detection of various histological markers of chromaffin cells (**Fig 3.8**). *TH*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ were born in normal mendelian ratios and exhibited no gross defects in histology of neonatal adrenal glands as determined by Sf1 and TH expression, as well as the cytoskeletal protein neuronal class III-β-tubulin (TuJ1) (**Fig 3.8A**). The expression of phenylethanolamine-N-methyltransferase (PNMT), an enzyme that catalyzes the conversion of norepinephrine to epinephrine and is expressed in virtually every chromaffin cell of the mouse medulla, was unchanged in *TH*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ mice compared to *TH*$^{+/+}$:*R26R*$^{+/DNMaml}$ mice [3, 44, 45]. Dopamine transporter 1 (DAT1), a membrane transporter protein responsible for reuptake of catecholamine hormones back into chromaffin cells, also exhibited no differences in expression [2, 46]. At P20, no differences in Sf1/TH and PNMT expression were also detected (**Fig 3.8C**). Furthermore, expression of 20α-hydroxysteroid dehydrogenase (20αHSD), a marker of the fetal adrenal cortex, was unchanged, which suggests that there is no effect on the development of the adrenal cortex.

We wondered if doubling the dosage of the DNMaml gene would be sufficient to compensate for the ineffectual Cre recombination, but no changes in TH, PNMT, or TuJ1 were detected in *TH*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ mice compared to *TH*$^{+/+}$:*R26R*$^{+/DNMaml}$ mice (**Fig 3.8B**). Since Notch signaling is known to modulate its own expression, we examined the expression of Dll4 at P20 but observed no changes. These data suggest that inhibition of Notch signaling in a subset of chromaffin cells has no effect on the gross histology of the adrenal medulla or

cortex. This may be a consequence of the lack of complete penetrance of the $TH^{+/Cre}$ line in mediating recombination. Older animals have also not been considered histologically.

To further examine the molecular phenotype of the medulla in the Notch inhibition mice, we performed QPCR analysis on adrenals from P12 $TH^{+/Cre}$:$R26R^{DNMaml/DNMaml}$ and $TH^{+/+}$:$R26R^{+/DNMaml}$ mice (**Fig 3.9A**). We identified a statistically significant downregulation in Hes1 in both animals analyzed and a statistically significant downreulation of Hes5 in one animal, which suggests that Notch signaling is indeed being inhibited. Of particular interest, we saw significant upregulation in TH expression. Furthermore, modest but statistically significant increases in expression of noradrenaline tranporter 1 (NAT1), a catecholamine reuptake transporter in the same family as DAT1 [46, 47], and chromogranin A, a peptide hormone secreted concurrently with catecholamine hormones [48], were identified. We also observed downregulations in DAT1 and the catechol-O-methyl transferase (COMT), an enzyme involved in conversion of catecholamines to excretable metabolites [49]. TH upregulation was confirmed by immunoblot analysis of adrenals from 3wks $TH^{+/+}$:$R26R^{+/DNMaml}$, $TH^{+/Cre}$:$R26R^{+/DNMaml}$ and $TH^{+/Cre}$:$R26R^{DNMaml/DNMaml}$ animals (**Fig 3.9B**). Quantification of the immunoblots identified increased levels of TH protein in 2/3 of the animals analyzed (**Fig 3.9C**). Taken together, these data suggest inhibition of Notch signaling may alter the catecholamine production, but not the gross histology, of chromaffin cells. Additional animals need to be analyzed in order to verify these results.

Overactivation of Notch signaling in TH+ cells results in a reduction of PNMT expression in chromaffin cells

To complement the Notch inhibition studies, we constitutively activated canonical Notch signaling in TH+ cells using the *R26R*$^{+/NICD}$ line (**Fig 3.10**). *R26R*$^{+/NICD}$ is a *Rosa26* locus knock-in mouse that employs a NeoSTOP cassette upstream of a sequence encoding a constitutively active NICD followed by an IRES-EGFP (**Fig 1E**) [27]. In the presence of Cre recombinase expression, the NeoSTOP cassette is excised and the NICD is expressed.

No differences in the gross histology of P0 and P20 adrenals from *TH*$^{+/Cre}$:*R26R*$^{+/NICD}$ compared to *TH*$^{+/+}$:*R26R*$^{+/NICD}$ was observed, as determined by immunofluorescent detection of Sf1 costained with TH and TH costained with 20αHSD (**Fig 3.10A**). However, at 6wks, the expression of PNMT, which normally exhibits a broad pattern of expression throughout the adrenal medulla, was detected as a salt-and-pepper expression pattern (**Fig 3.10B**). PNMT- cells were clearly detected in *TH*$^{+/Cre}$:*R26R*$^{+/NICD}$ compared to *TH*$^{+/+}$:*R26R*$^{+/NICD}$ mice, which suggests that the population of epinephrine-secreting adrenergic chromaffin cells is reduced with overactivation of Notch signaling. The expression of DAT1 and TuJ1 showed no difference at this time point.

This observation was confirmed through QPCR analysis of adrenals from 8wks *TH*$^{+/Cre}$:*R26R*$^{+/NICD}$ and *TH*$^{+/+}$:*R26R*$^{+/NICD}$ mice (**Fig 3.10C**). We identified a statistically significant increase in Hes1 and Hes5 expression in one animal

analyzed concurrent with a downregulation of PNMT. Upregulations in aromatic amino acid decarboxylase (AADC) and dopamine-β-hydroxylase (DBH), two enzymes that precede PNMT in epinephrine synthesis [2], were also observed. Taken together, these data suggest overactivation of Notch signaling may reduce PNMT expression and drive chromaffin cells towards a noradrenergic fate. However, only one of the two animals analyzed exhibited this phenotype by QPCR. These observations need to be confirmed with a larger sample size.

Canonical Notch signaling is dispensable for development of the adrenal cortex but not for the ovary

Canonical Notch signaling does not appear to be occurring the adrenal cortex (**Fig 3.2**) although Jag1 expression can be detected in the subcapsular region (**Fig 3.4D**). We have previously reported an upregulation of Jag1 in adrenocortical carcinoma, which mediates a non-cell autonomous effect on cell proliferation through canonical Notch signaling (**Chapter 2** of this thesis and[50]). To interrogate whether or not active Notch signaling is occurring in the normal adrenal cortex, we employed the $Sf1^{+/Cre}$ line crossed to the $R26R^{+/DNMaml}$ line. The $Sf1^{+/Cre}$ is a transgenic mouse driving Cre expression from the complete Sf1 promoter (**Fig 1F**) [24]. We have previously reported the successful function of this line in our hands [29].

To assess the function of Notch in Sf1+ cells, we analyzed adrenals from male and female $Sf1^{+/Cre}$:$R26R^{+/DNMaml}$ and $Sf1^{+/Cre}$:$R26R^{DNMaml/DNMaml}$ mice (**Fig**

3.11 and **Fig 3.12**). Histological analysis revealed no gross differences in the adrenals from *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ mice compared to *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$ mice for both females (8-65wks of age, **Fig 3.11A**) and males (18-50wks of age, **Fig 3.12A**). Immunofluorescent analysis of TH and Cyp11b1, a key enzyme in cortisol synthesis, in 35wks females, revealed no gross differences in medulla and cortex respectively (**Fig 3.11C**). Sf1 costained with TH at 50wks of age confirmed the lack of apparent phenotype in both females (**Fig 3.11D**) and males (**Fig 3.12B**). We have previously shown that β-catenin is required for adrenocortical development and maintenance and its expression is restricted to the subcapsular zone [29]. We analyzed β-catenin expression at 50wks in females (**Fig 3.11D**) and males (**Fig 3.12B**) but did not identify any differences in expression, which suggests that adrenocortical stem/progenitor function is unaffected. Finally, QPCR analysis of adrenals from 35wks *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$, and *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$ confirmed that Notch target genes were not affected nor histological markers Sf1 and TH (**Fig 3.11B**). Taken together, these data show that Notch signaling is dispensable for development and maintenance of the adrenal cortex.

In addition to the adrenal cortex, Sf1 expression also is required for the gonads [51]. Indeed, the *Sf1*$^{+/Cre}$ line has previously been shown to successfully target Sf1+ cells of the gonad in addition to the adrenal cortex[24]. One explanation for the lack of phenotype in Sf1$^{+/Cre}$:*R26R*$^{+/DNMaml}$ mice could be lack of recombination but this is clearly not the case as ovaries collected from these mice exhibit severe abnormalities (**Fig 3.13**). Histological analysis of ovaries from

Sf1$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMam}$ females reveals multi-oocytic follicles and the development of blood-filled cysts compared to normal, single-oocytic follices (**Fig 3.13A**). Interestingly, ovaries from younger mice appear histologically similar but the phenotype becomes severe by 30wks of age. There did not appear to be difference in expression of Sf1 in ovaries from *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ compared to *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$ females, which suggest the defect may lie in maturation and/or release of follicles during ovulation (**Fig 3.13B**).

Overactivation of Notch signaling in Sf1+ cells results in stochastic adrenal aplasia

Because we have reported an activation of Notch signaling in adrenal tumors, we also overactivated Notch signaling in Sf1+ by crossing the *Sf1*$^{+/Cre}$ line to the *R26R*$^{+/NICD}$ line (**Fig 3.14**). Interestingly, adrenal aplasia was identified in *Sf1*$^{+/Cre}$:*R26R*$^{+/NICD}$ mice, as determined by whole-organ analysis of the urinary tract (**Fig 3.14A, B**). Adrenal progenitor cells appeared to migrate the proper location, superior to the kidneys, but were severely reduced in size. About 50% of animals died at birth, most likely due to a loss of hormones secreted from the adrenal cortex and/or medulla. Immunofluorescent analysis revealed a stochastic effect on the Sf1+ cortical cells and TH+ medullary cells. (**Fig 3.14C**). In some cases, the aplastic adrenal appeared to be composed mostly of Sf1+ cells while in other animals, the aplastic adrenal was composed mostly of TH+ cells.

Because of the fact that Notch inhibition has no effect on the development of the adrenal cortex, strongly suggests that Notch is inactive in Sf1+ cells. Therefore, interpretation of the results in the *Sf1*$^{+/Cre}$:*R26R*$^{+/NICD}$ mice and was pursued further. Taken together with the *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ studies, these data suggest that Notch signaling is dispensable for development of the adrenal cortex and may even be actively repressed.

Discussion

Active Notch signaling is occurring in chromaffin cells of the adrenal medulla as determined by the expression of the canonical Notch target genes Hes1 and Hes5, the canonical Notch ligands Jag1 and Dll4, the noncanonical ligand Dlk1, and the Notch receptors Notch1 and Notch2. Hes1 and Hes5 expression are also present in TH+ chromaffin progenitors pre- and post-invasion of the adrenal primordia. Inhibition of canonical Notch signaling does not result in gross histological changes in chromaffin cells but results in an upregulation of the catecholamine-synthesizing enzyme TH at the mRNA and protein level. In contrast, overactivation of Notch signaling in TH+ cells results in reduced expression of epinephrine-synthesizing enzyme PNMT, which suggests there may be reduction in the population of adrenergic chromaffin cells. Furthermore, inactivation of Notch signaling in Sf1+ cells has no effect on adrenocortical development or maintenance but results in severe abnormalities in the ovary. Overactivation of Notch signaling in Sf1+ cells results in adrenal aplasia.

Chromaffin cells are derived from the SA lineage of the neural crest. In addition to the medullary chromaffin cells, the SA lineage gives rise to sympathetic ganglia and sites of extra-adrenal paraganglia such as the suprarenal ganglia and the fetal “organ of Zuckerkandl” [10]. While numerous transcription factors have been implicated in SA differentiation, the signals that define a chromaffin versus a sympathetic fate are ill defined. Furthermore, molecular signals that are required for chromaffin progenitor cell invasion into the adrenal primordial and maturation in the fetal adrenal environment are poorly understood. The observation that many Hes1+ (GFP+) cells that lack TH expression aggregate at the vicinity of the adrenal primordia is intriguing. It is unlikely that these TH-/GFP+ cells are Sf1+ fetal adrenocortical cells since we have demonstrated that canonical Notch signaling is dispensable for adrenocortical development. Several studies have suggested that some chromaffin progenitors that migrate into the adrenal primordial lack TH expression until post-migration [9, 52]. Therefore, Hes1 may define a population of pluripotent SA progenitors that is not committed to a chromaffin cell fate until after cessation of invasion of the adrenal primordia.

Furthermore, the adrenal medulla has been analyzed in studies where transcription factors implicated in early SA progenitor differentiation, such as Mash1 and Phox2B, have been ablated. In Mash1 deficient mice, expression of downstream transcription factors dHand and Phox2A are not disrupted in a majority of chromaffin cells, which suggests a Mash1-independent population of chromaffin cells invade the adrenal medulla [4]. In contrast, in Phox2B deficient

mice, dHand and Phox2A were lost in a majority of chromaffin cells but Mash1 expression was still detectable in some TH+ cells [5]. These studies suggest chromaffin progenitors dependent upon Mash1 are independent of chromaffin progenitors dependent on Phox2B. The developmental origin of these distinct populations and there relationship to SA progenitors of the sympathetic lineage is not clear. Taken together, these studies suggest that a single population of SA progenitors committed to a chromaffin cell fate may not invade the adrenal primordial. Notch signaling therefore may serve as a common signal for SA progenitors that express differential transcription factor profiles (i.e. Mash1 or Phox2B).

Indeed, expression of the noncanonical Notch ligand Dlk1 has been identified in chromaffin progenitors prior to invasion of the adrenal anlage, during maturation of chromaffin cells in the fetal adrenal gland, and in fully differentiated adult chromaffin cells [41, 42, 53]. However, when we inhibited Notch signaling in TH+ cells, the adrenal medulla developed normally and there was no evidence that chromaffin cells were not differentiated. Two potential explanations for these results seem plausible. First, the lack of penetrance of the $TH^{+/Cre}$ suggests a majority of the SA progenitors are not being targeted and the remaining population may be sufficient for the development of the medulla. Second, as discussed above, a subpopulation of the chromaffin progenitor cells that invade the adrenal anlage may not express TH until after they have taken up residence within the fetal adrenal gland [9]. Therefore, inhibition of Notch signaling in TH+

cells would have no effect on this population. Further studies are required to elucidate these possibilities.

However, the role of Notch signaling in the SA lineage and chromaffin progenitors may be distinct from its function in mature chromaffin cells of the adult adrenal medulla. In our Notch inhibition studies, we identified an upregulation of TH mRNA and protein concurrent with upregulations in the message of NAT1 and ChrA and downregulations of COMT and DAT1. The observations that TH is upregulated and COMT is downregulated suggests an increase in catecholamine production may be occurring. The altered expression of the reuptake transporters DAT1 and NAT1 further supports this hypothesis but since both transporters can reuptake epinephrine and norepinephrine, the specific changes in catecholamine production cannot be determined from gene expression changes alone [46]. The possibility of changes in the catecholamine profile secreted from chromaffin cells in $TH^{+/Cre}$:$R26R^{DNMaml/DNMaml}$ animals needs to be confirmed with analysis of circulating catecholamine levels and/or their metabolites (metanephrines) [54].

The proper dosage of Notch signaling may also be important in defining noradrenergic (norepinephrine-secreting) and adrenergic (epinephrine-secreting) chromaffin cell types. In our Notch overactivation studies we observed a loss of PNMT expression in 6wks $TH^{+/Cre}$:$R26R^{+/NICD}$ animals as shown by immunfluorescent detection of PNMT protein and QPCR analysis of PNMT mRNA. These data suggest that an excess of Notch signaling may drive chromaffin cells towards a noradrenergic cell fate. However, this phenotype has

only been examined in adult mice and was not 100% penetrant amongst all animals analyzed, possibly due to the ineffectual *TH*$^{+/Cre}$ line. Additional studies of aged *TH*$^{+/Cre}$:*R26R*$^{+/NICD}$ are required to confirm these observations.

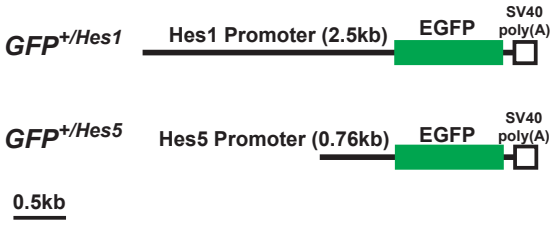
Separate from the observations in the adrenal medulla, we have shown that Notch signaling is dispensable for the development and maintenance of the adrenal cortex. In both male and female *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMam}$ mice, the adrenal cortex and medulla formed normally. Furthermore, expression of the cortisol-synthesizing enzyme Cyp11b1 was unchanged in Notch inhibition mice. Additionally, expression of the Wnt pathway downstream effector β-catenin, which is believed to be involved in maintenance of adrenocortical stem/progenitors cells, was unchanged [29, 38, 39]. Conversely, overactivation of Notch signaling in Sf1+ cells resulted in adrenal aplasia, but since Notch signaling is not active in Sf1+ cells, the interpretation of these results is difficult. Taken together, these studies suggest an absence of Notch signaling in Sf1+ cells is required for adrenocortical development.

Interestingly, ovaries in female *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ mice developed multi-oocytic follicles and blood-filled cysts. Previous reports have shown that Notch pathway genes are expressed in ovarian follicles and pharmacologic inhibition of Notch signaling results in reduced numbers of primordial follicles, precursors to follicles that develop with the onset of sexual maturity [55, 56]. Our studies further support the importance of Notch signaling in follicle development but, because our lab does not study the gonads, this hypothesis was not pursued.

The observations in the Notch overactivation studies may have implication for pheochromocytoma (PCC), a catecholamine-secreting neoplasm that is derived from adrenal medullary chromaffin cells [57, 58]. Several hereditary cancer syndromes have been identified that manifest in PCC. PCC develop in about 10-20% of the autosomal dominant von Hippel-Lindau (vHL) syndrome and these PCC primarily secrete norepinephrine [59, 60]. In contrast, PCC are detected in about 50% of patients with autosomal dominant syndrome multiple endocrine neoplasia type 2 (MEN2) and these PCC are primarily epinephrine-secreting [60-62]. Gene expression profiling studies have identified an upregulation of Jag1 and Notch3 in vHL and sporadic norepinephrine-secreting PCC compared to MEN2 and sporadic epinephrine-secreting PCC [63]. Chromaffin cells in our Notch overactivation studies exhibit reductions in PNMT expression, which suggests that chromaffin cells in this model adopt a noradrenergic-like phenotype. Taken together, these data suggest an upregulation of Notch signaling in PCC may define an norepinephrine-favored catecholamine secretory profile. Furthermore, Notch signaling has been shown to be downregulated in malignant and recurring PCC, which suggests that Notch may confer a more differentiated phenotype in some PCC [64, 65]. These observations correlate with our identification of Notch ligand, receptor, and target gene expression in mature chromaffin cells. Targeting inhibition of Notch signaling may therefore have dual effects in PCC depending on the catecholamine secretory profile and the aggressiveness of the PCC tumor but further studies are needed to elucidate this potential therapeutic avenue.

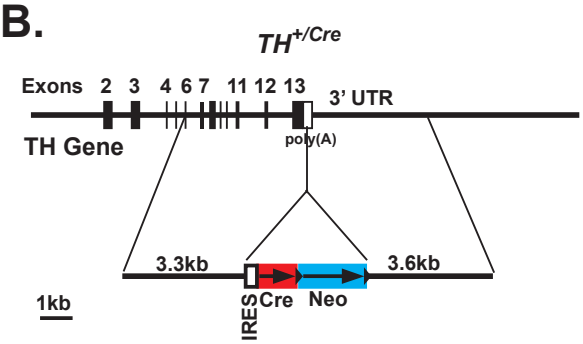
Figure 3.1: Mouse lines. A. Transgenic *Hes1*$^{+/GFP}$ and *Hes5*$^{+/GFP}$ mice in which EGFP expression is driven by 2.5kb and 0.76kb of the Hes1 and Hes5 promoters, respectively. The SV(40) poly(A) signal is used for proper translation of EGFP. **B.** The *TH*$^{+/Cre}$ line is knock-in of IRES-Cre recombinase in the 3' UTR of the mouse *TH* gene. Arrows indicate orientation of the Cre cassette. Triangles indicate Frt sites used for excision of the Neo cassette following selection of positive clones. **C.** The *R26R-Tom*$^{+/EGFP}$ line employs expression of Tomato protein driven by the pCA promoter and is a knock-in into the *Rosa26* locus. In the presence of Cre recombinase expression, the Tomato cassette is excised (triangles indicate LoxP sites) and EGFP protein is expressed. **D.** The *R26R*$^{+/DNMaml}$ line employs a NeoSTOP cassette upstream of the DNMaml-EGFP fusion protein cassette and is knocked into the *Rosa26* locus. In the presence of Cre recombinase, the NeoSTOP cassette is excised (triangles indicate LoxP sites) and the DNMaml protein is expressed. The Poly(A) sequence is indicated. **E.** The line employs a NeoSTOP cassette upstream of the NICD-IRES-EGFP cassette and is knocked into the *Rosa26* locus. In the presence of Cre recombinase, the NeoSTOP cassette is excised (triangle indicate LoxP sites) and the NICD-IRES-EGFP protein is expressed. The Poly(A) sequence is indicated. **F.** The transgenic *Sf1*$^{+/Cre}$ uses a 111kb BAC clone which contains the complete Sf1 promoter and coding region (top panel). Cre recombinase is inserted into upstream of Sf1 exons 2 and 3 (bottom panel). Four copies of the final Sf1-Cre BAC clone is incorporated into the genome in a random location.

A.



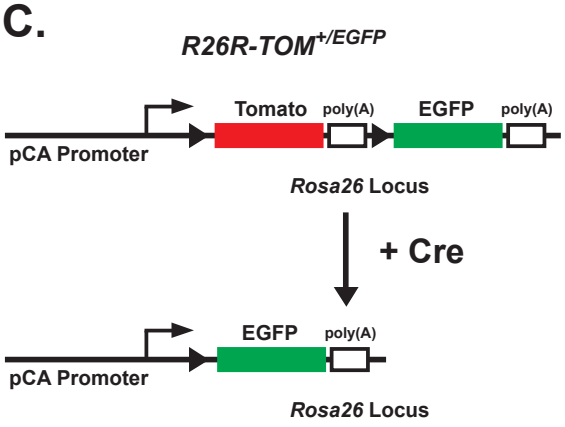
(Adapted from Ohtsuka T et al. MCN. 31: 109-122. 2006)

B.



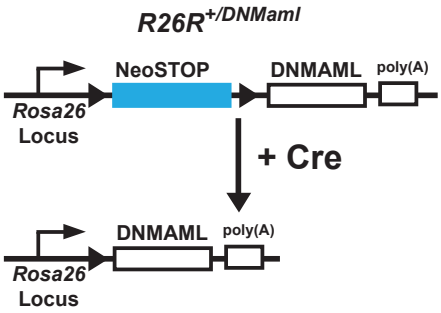
(Adapted from Lindenberg J et al. *Genesis*. 40: 67-73. 2004)

C.



(Adapted from Muzumdar MD et al. *Genesis*. 45: 593-605. 2007)

D.



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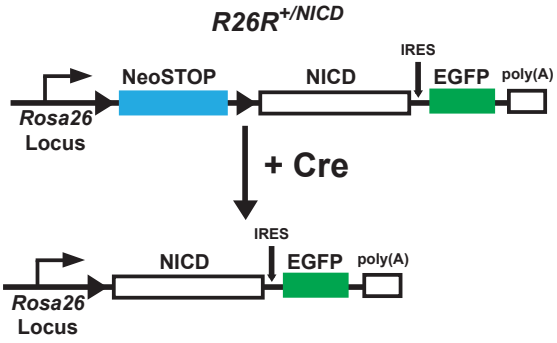
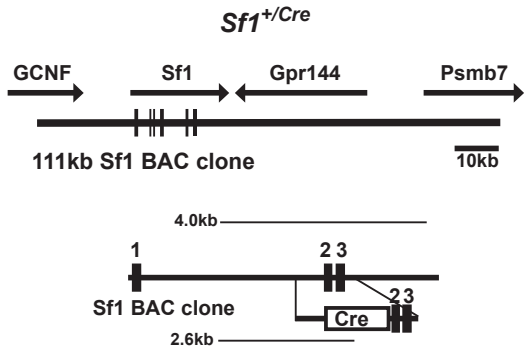
E.



(Adapted from Murtaugh CL et al. *PNAS*. 100(25): 14920-14925. 2003)

F.



(Adapted from Bomgham NC et al. *Genesis*. 44: 419-424. 2006)

Figure 3.2: Canonical Notch target genes Hes1 and Hes5 are expressed in chromaffin cells of the adrenal medulla. A. Immunofluorescence of *Hes1*$^{GFP/GFP}$ male mice at P0, 4, 12, and 23 weeks of age for GFP (red) or GFP (red) co-stained with TH (green). Immunofluorescence for Sf1 (red) co-stained with TH (green) is used to indicate the adrenal cortex (c) and medulla (m). Dapi is used as counterstain. **C.** Immunofluorescence of *Hes5*$^{GFP/GFP}$ male mice at P5 and 4 weeks of age for GFP (red) or GFP (red) co-stained with TH (green). Immunofluorescence for Sf1 (red) co-stained with TH (green) are used to indicate the adrenal cortex (c) and medulla (m). Dapi is used as counterstain.

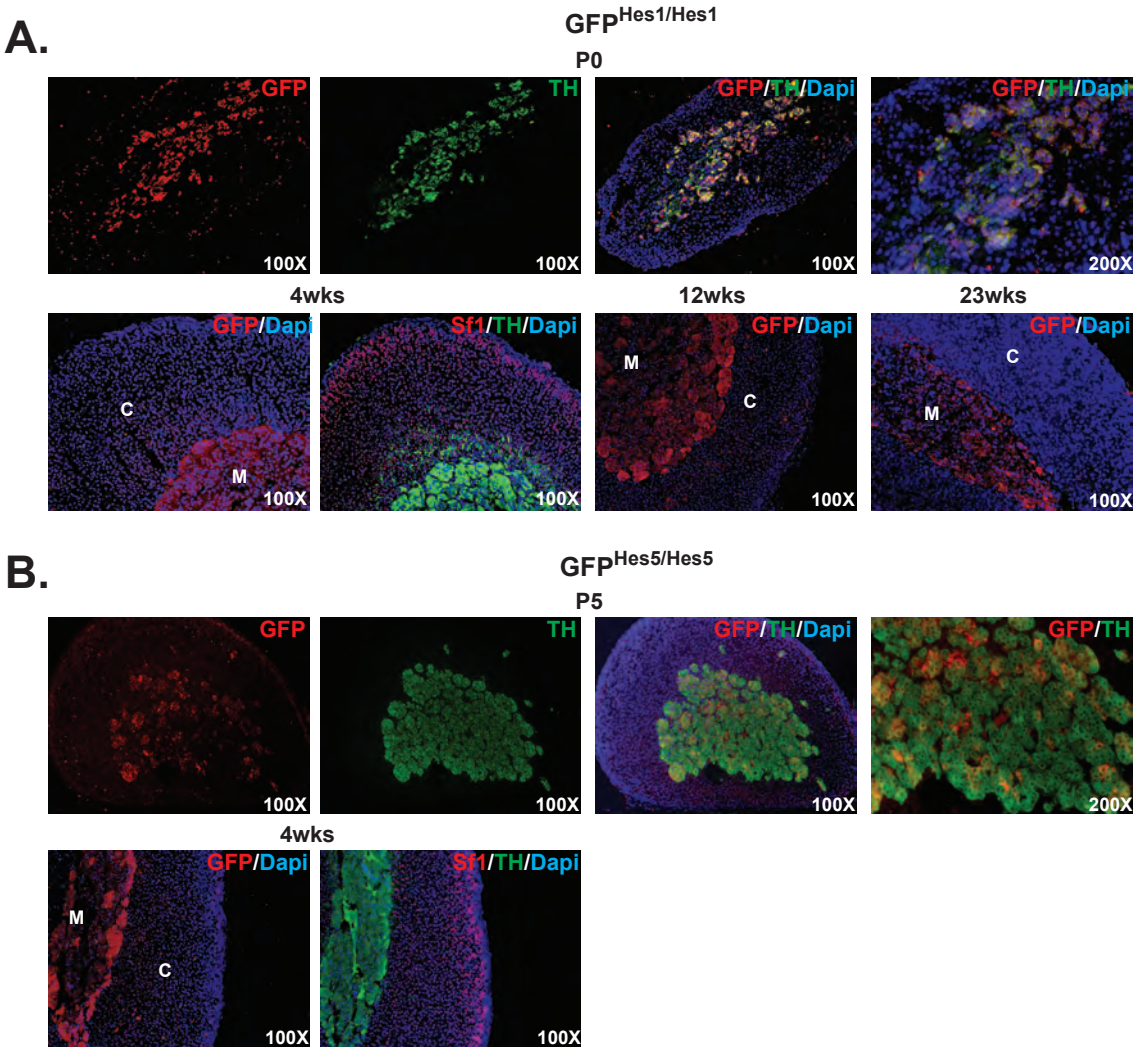
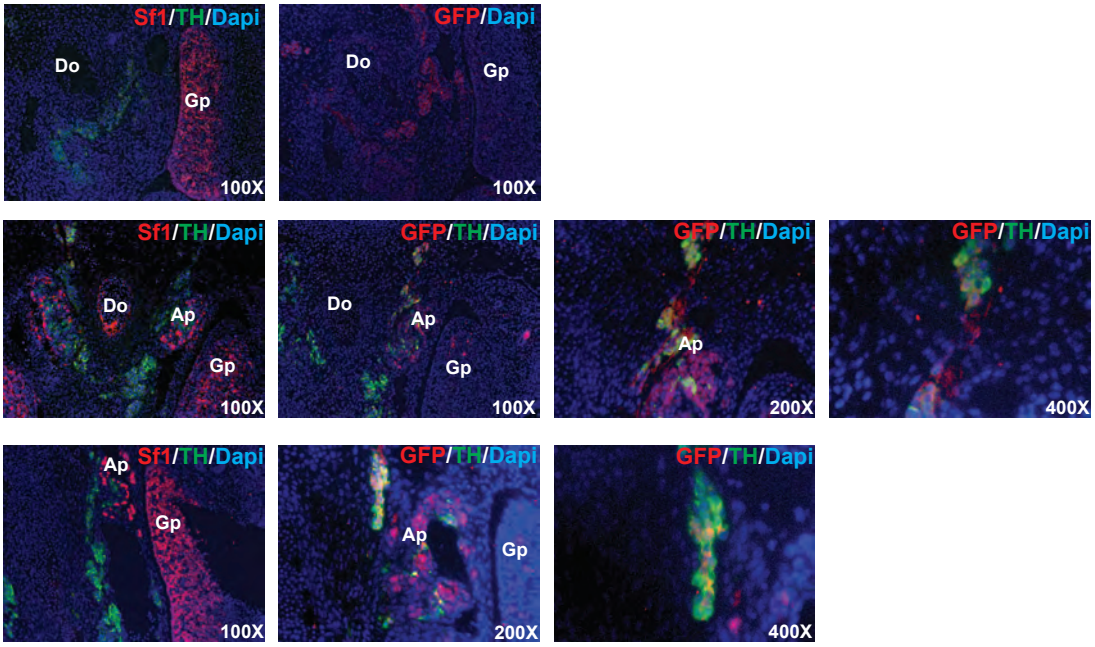
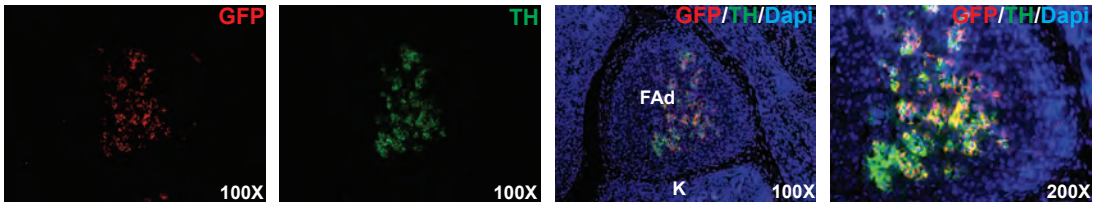

A.
GFP Hes1/Hes1
P0
GFP
100X
TH
100X
GFP/TH/Dapi
100X
GFP/TH/Dapi
200X
4wks
12wks
23wks
GFP/Dapi
C
M
100X
Sf1/TH/Dapi
100X
GFP/Dapi
M
C
100X
GFP/Dapi
C
M
100X
B.
GFP Hes5/Hes5
P5
GFP
100X
TH
100X
GFP/TH/Dapi
100X
GFP/TH
200X
4wks
GFP/Dapi
M
C
100X
Sf1/TH/Dapi
100X

Figure 3.3: Canonical Notch target genes Hes1 and Hes5 are expressed in chromaffin progenitor cells during development. A. Immunofluorescence of E12.5 *Hes1*$^{GFP/GFP}$ male mice for GFP (red) co-stained with TH (green). Immunofluorescence for Sf1 (red) co-stained with TH (green) are used to indicate the adrenal primordium (Ap) and Gonadal primordium (Gp). Images are oriented Doral (top) to ventral (bottom). Dorsal aorta: Do. Dapi is used as counterstain. **B.** Immunofluorescence of E14.5 *Hes5*$^{GFP/GFP}$ mice for GFP (red) co-stained with TH (green). The fetal adrenal gland (Fad) and kidney (K) are indicated. Dapi is used as counterstain. **C.** Immunofluorescence of E16.5 *Hes1*$^{GFP/GFP}$ mice for GFP (red). Dapi is used as counterstain.

A. E12.5 *GFP*$^{Hes1/Hes1}$



B. E14.5 *GFP*$^{Hes5/Hes5}$



C. E16.5 *GFP*$^{Hes1/Hes1}$

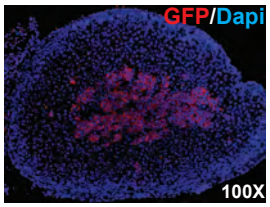


Figure 3.4: Notch ligands Jag1 and Dll4 are expressed in the mouse adrenal medulla. A. Efficiency-corrected ΔCT QPCR of mRNA in triplicate samples used to quantify the expression of the five Notch ligands in WT male (left panel; time points 0, 3, 7, 18, 40 and 50 weeks of age, n=1 for each time point) and Female (right panel; time points 0, 3, 8, 18, 27, 46 weeks of age, n=1 for each time point) mice. Normalized to β -actin. **B.** Relative expression QPCR of mRNA from triplicate samples for the five Notch ligands in WT male (left panel; time points 0, 3, 7, 18, 40, 48, 50 weeks of age, n=1 for each time point) and WT female (right panel; time points 0, 3, 8, 18, 27, 31, 38, 46 weeks of age, n=1 for each time point) mice. Normalized to β -actin. **C.** Immunofluorescence of adrenal glands from male mice for Dll4 (red) or Dll4 (red) co-stained with TH (green) at 3, 12, 23, and 50 weeks of age. Dapi is used as counterstain. **D.** Immunofluorescence of adrenal glands from male mice for Jag1 (red) at 23 and 48 weeks of age. Dapi is used as counterstain.

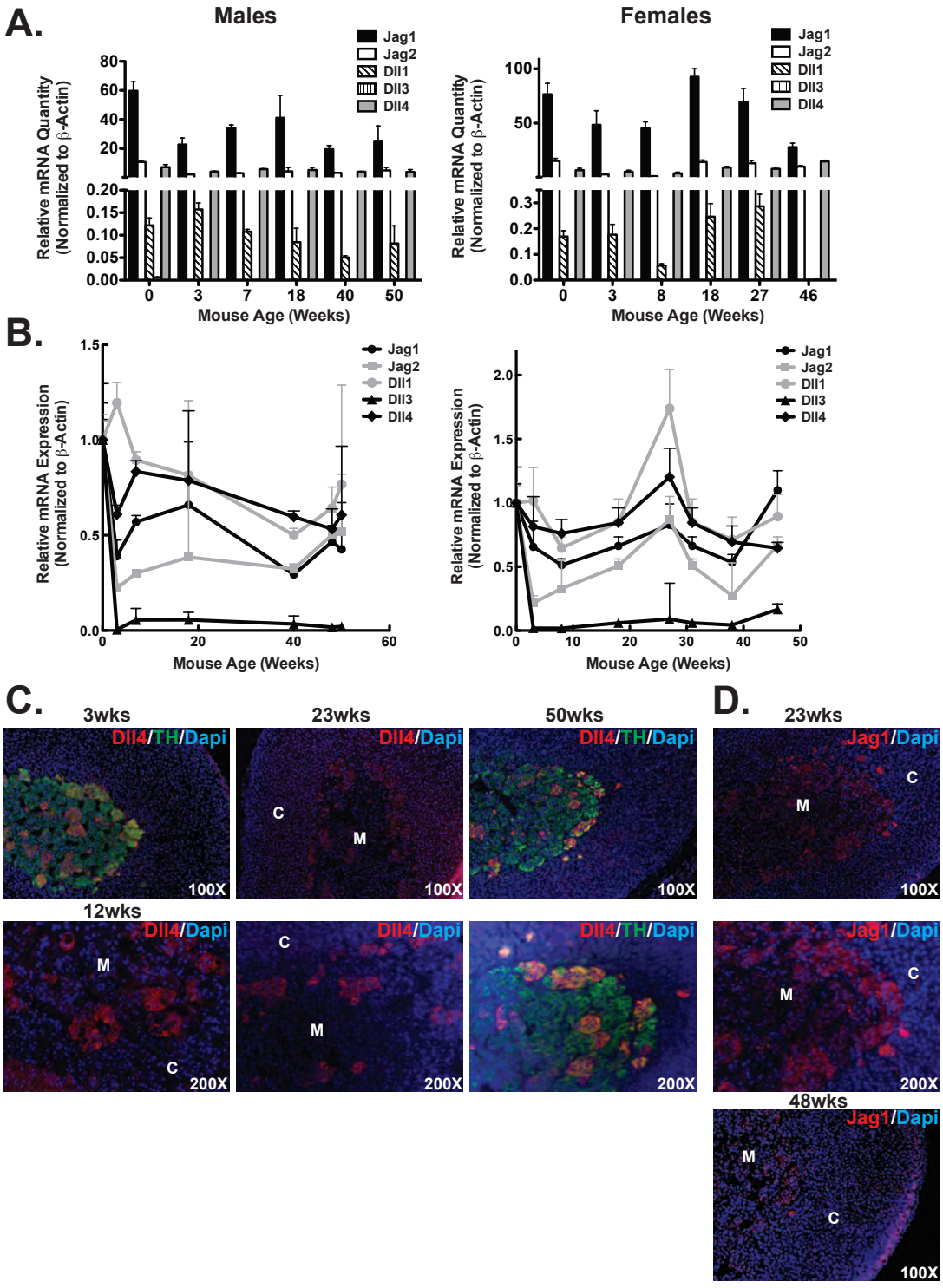

A.
Males
Females
Jag1
Jag2
Dll1
Dll3
Dll4
Relative mRNA Quantity (Normalized to β-Actin)
Mouse Age (Weeks)
B.
Relative mRNA Expression (Normalized to β-Actin)
C.
3wks
23wks
50wks
12wks
Dll4/Dapi
Dll4/TH/Dapi
C
M
100X
200X
D.
23wks
48wks
Jag1/Dapi

Figure 3.5: The non-canonical Notch ligand Dlk1 is expressed in TH positive chromaffin cells. Immunofluorescence for Dlk1 (Red), TH (green) and Dapi (blue) as a counterstain at E14.5 (**A.)**, P0 (**B.)**, 18 and 27 weeks (**C.)** of age. The cortex (c) and medulla (m) are indicated in images where staining for TH was not performed.

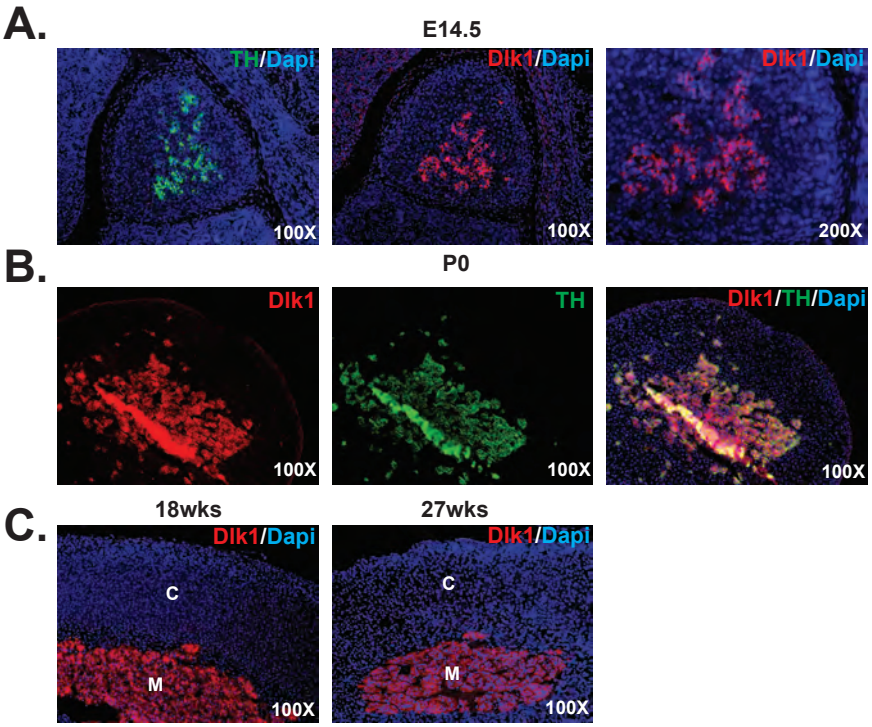

A.
E14.5
TH/Dapi
100X
Dlk1/Dapi
100X
Dlk1/Dapi
200X
B.
P0
Dlk1
100X
TH
100X
Dlk1/TH/Dapi
100X
C.
18wks
Dlk1/Dapi
C
M
100X
27wks
Dlk1/Dapi
C
M
100X

Figure 3.6: Notch receptors Notch1 and Notch2 are expressed in the mouse adrenal medulla. A. Efficiency-corrected ΔCT QPCR of mRNA in triplicate samples used to quantify the expression of the four Notch receptors in WT male (left panel; time points 0, 3, 7, 18, 40 and 50 weeks of age, n=1 for each time point) and Female (right panel; time points 0, 3, 8, 18, 27, 46 weeks of age, n=1 for each time point) mice. Normalized to β-actin. **B.** Relative expression QPCR of mRNA from triplicate samples for the four Notch receptors in WT male (left panel; time points 0, 3, 7, 18, 40, 48, 50 weeks of age, n=1 for each time point) and WT female (right panel; time points 0, 3, 8, 18, 27, 31, 38, 46 weeks of age, n=1 for each time point) mice. Normalized to β-actin. **C.** Immunofluorescence of adrenal glands from male mice for Notch2 (red) or Notch2 (red) co-stained with TH (green) at 23 and 40 weeks of age. Dapi is used as counterstain. **D.** Immunofluorescence of adrenal glands from male mice for Notch1 (red) at 23 weeks of age. Dapi is used as counterstain.

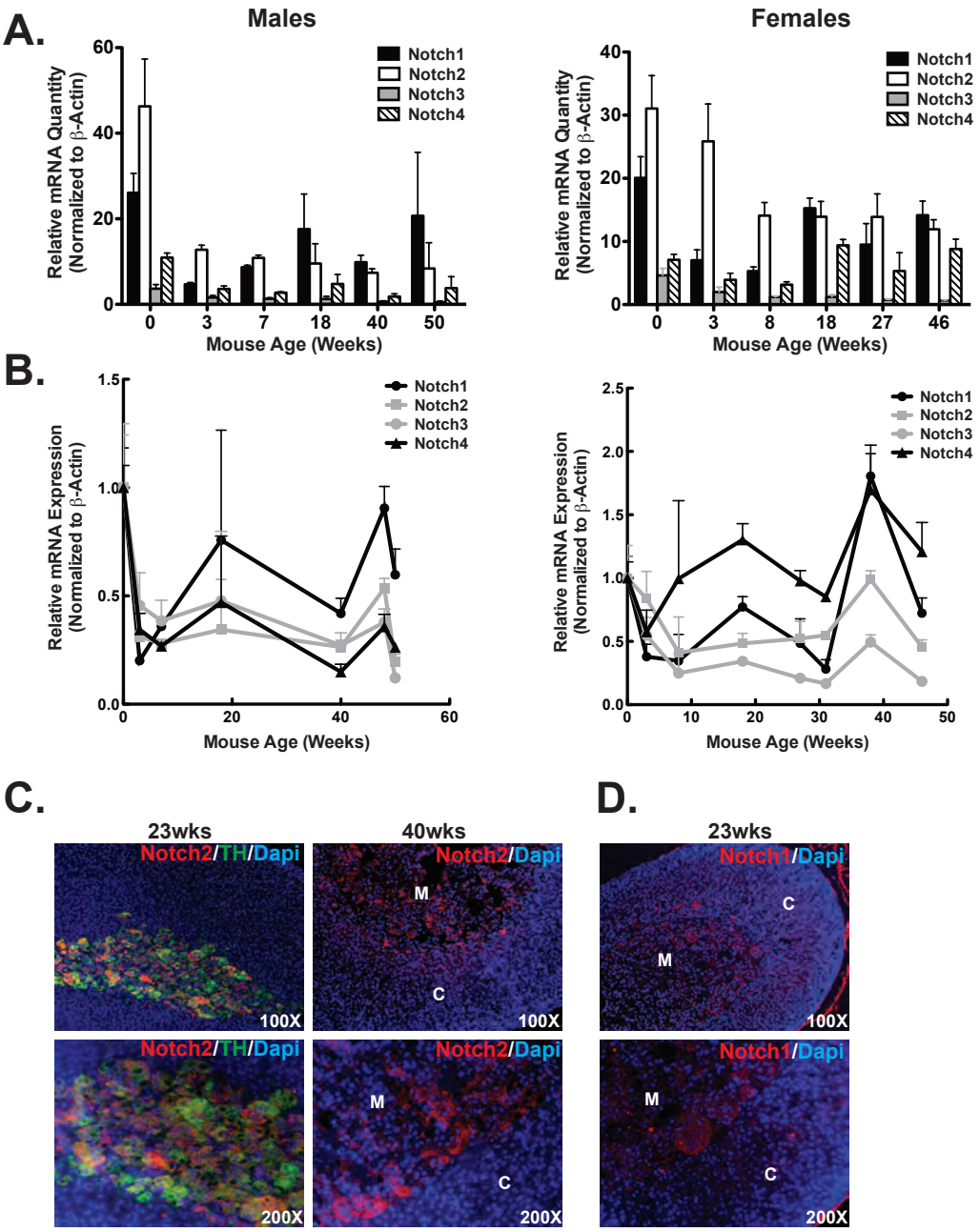

A.
Males
Females
Relative mRNA Quantity (Normalized to β-Actin)
Notch1
Notch2
Notch3
Notch4
Mouse Age (Weeks)
B.
Relative mRNA Expression (Normalized to β-Actin)
C.
23wks
40wks
Notch2/TH/Dapi
Notch2/Dapi
M
C
100X
200X
D.
Notch1/Dapi

Figure 3.7: Targeting chromaffin cells of the adrenal medulla using *TH$^{+/Cre}$* mouse line. Immunofluorescence for GFP (red) and TH (green) in *TH$^{+/+}$*:*R26R-Tom$^{+/EGFP}$* (left panels) and *TH$^{+/Cre}$*:*R26R-Tom$^{+/EGFP}$* (right panels). Dapi is used as counterstain. Each row represents a cross with a different *TH$^{+/Cre}$* mouse.

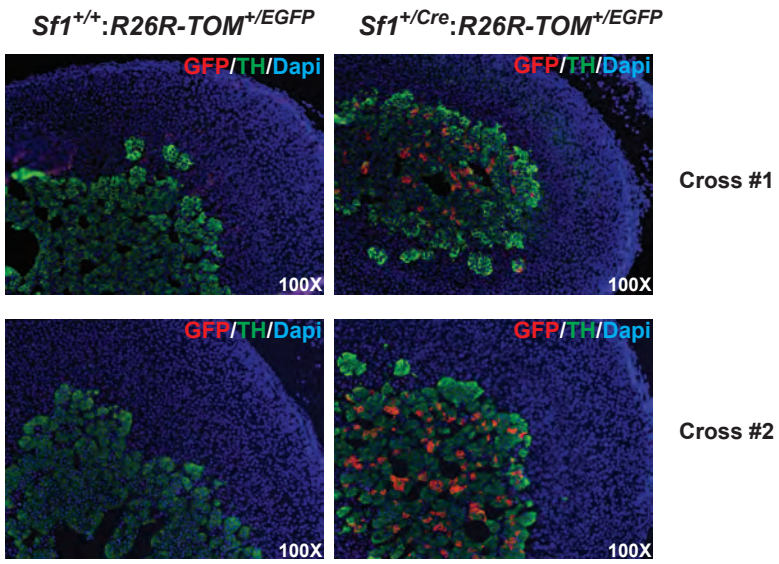

Sf1+/+:R26R-TOM+/EGFP
Sf1+/Cre:R26R-TOM+/EGFP
GFP/TH/Dapi
100X
Cross #1
Cross #2

Figure 3.8: Inhibition of canonical Notch signaling has no effect on the gross histology of the adrenal medulla. **A.** Immunofluorescence analysis of adrenals from P0 *TH*$^{+/+}$:*R26R*$^{+/DNMaml}$ and *TH*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ male mice for Sf1 (red) costained with TH (green), PNMT (red), DAT1 (red) and TuJ1 (red). Dapis is used as counterstain. **B.** Immunofluorescence analysis of adrenals from P12 *TH*$^{+/+}$:*R26R*$^{+/DNMaml}$ and *TH*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ male mice for TH (green) costained with PNMT (red) and TuJ1 (red). Dapi is used as counterstain. **C.** Immunofluorescence analysis of adrenals from P20 *TH*$^{+/+}$:*R26R*$^{+/DNMaml}$ and *TH*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ male mice for Sf1 (red) costained with TH (green), PNMT, 20αHSD (red) costained with TH(green), and Dll4 (red) costained with TH (green). Dapi is used as counterstain.

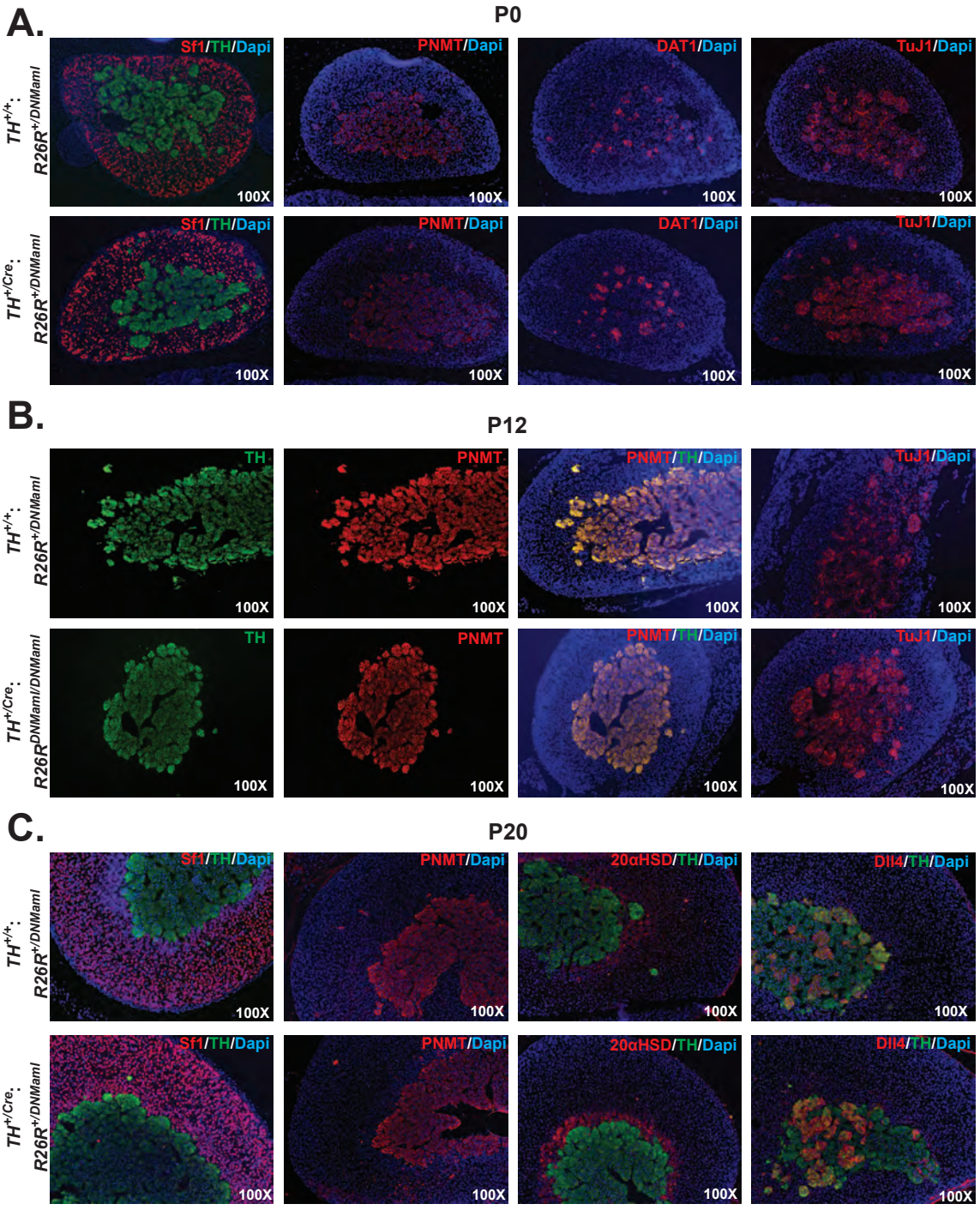

A.
P0
TH+/+: R26R+/DNMaml
TH+/Cre: R26R+/DNMaml
Sf1/TH/Dapi
PNMT/Dapi
DAT1/Dapi
TuJ1/Dapi
100X
B.
P12
TH+/+: R26R+/DNMaml
TH+/Cre: R26RDNMaml/DNMaml
TH
PNMT
PNMT/TH/Dapi
TuJ1/Dapi
100X
C.
P20
TH+/+: R26R+/DNMaml
TH+/Cre: R26R+/DNMaml
Sf1/TH/Dapi
PNMT/Dapi
20αHSD/TH/Dapi
Dll4/TH/Dapi
100X

Figure 3.9: TH mRNA and protein is upregulated in *TH$^{+/Cre}$:R26R$^{+/DNMaml}$* mice.

A. Relative expression QPCR (triplicate determinations) of mRNA from adrenals of P12 male *TH$^{+/+}$:R26R$^{+/DNMaml}$* (n=1) and *TH$^{+/Cre}$:R26R$^{DNMaml/DNMaml}$* (n=2) mice. ThCre/DNMaml/DNMaml #1 vs Wt/DNMaml * p<0.0001, ** p=0.03, ThCre/DNMaml/DNMaml #2 vs Wt/DNMaml, # p<0.004, ## p=0.05. **B.** Immunoblot analysis of adrenals from *TH$^{+/+}$:R26R$^{+/DNMaml}$* (n=1), *TH$^{+/Cre}$:R26R$^{+/DNMaml}$* (n=2), and *TH$^{+/Cre}$:R26R$^{DNMaml/DNMaml}$* (n=2) male mice at 3wks of age. **C.** Quantification of the immunblot analysis for TH protein levels normalized to β-actin.

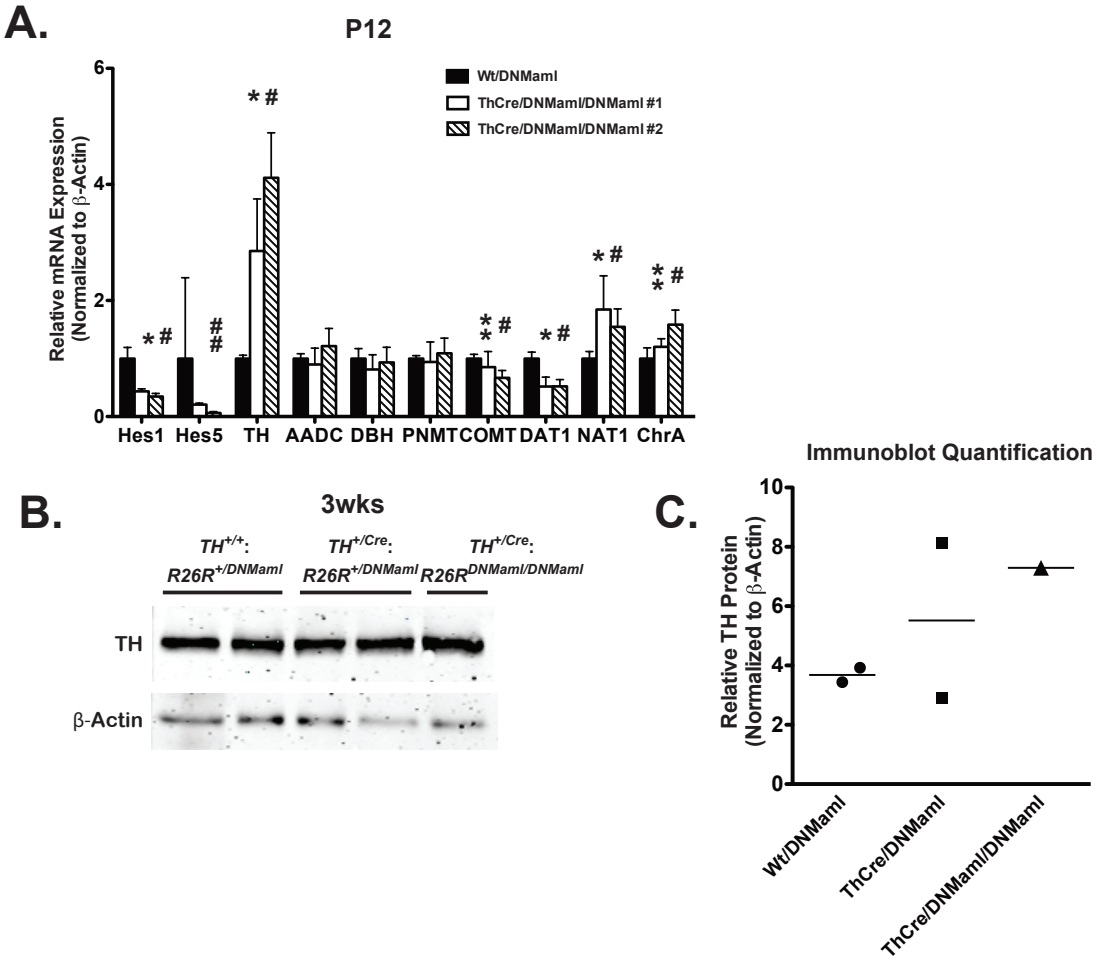

A.
P12
Wt/DNMaml
ThCre/DNMaml/DNMaml #1
ThCre/DNMaml/DNMaml #2
Relative mRNA Expression (Normalized to β-Actin)
Hes1
Hes5
TH
AADC
DBH
PNMT
COMT
DAT1
NAT1
ChrA
B.
3wks
TH+/+: R26R+/DNMaml
TH+/Cre: R26R+/DNMaml
TH+/Cre: R26RDNMaml/DNMaml
TH
β-Actin
C.
Immunoblot Quantification
Relative TH Protein (Normalized to β-Actin)
Wt/DNMaml
ThCre/DNMaml
ThCre/DNMaml/DNMaml

Figure 3.10: Overactivation of canonical Notch signaling reduces the population of adrenergic chromaffin cells. A. Immunofluorescence analysis of *Th*$^{+/+}$:*R26R*$^{+/NICD}$ and *TH*$^{+/Cre}$:*R26R*$^{+/NICD}$ male mice at P0 and P20 for Sf1 (red) costained with TH (green), 20αHSD costained with TH (green) and Dll4 (red) costained with TH (green). Dapi is used as counterstain. **B.** Immunofluorescence analysis of mice 6wks of age for TH (green), PNMT (red), DAT1 (red), and TuJ1 (red). Dapis is used as counterstain. **C.** Relative expression QPCR (triplicate determinations) of mRNA from adrenals of 8wks *Th*$^{+/+}$:*R26R*$^{+/NICD}$ (n=2) and *TH*$^{+/Cre}$:*R26R*$^{+/NICD}$ (n=2) male mice. * ThCre/NICD #1 vs Wt/NICD #1, $p<0.02$, # ThCre/NICD vs Wt/NICD #2, $p<0.02$.

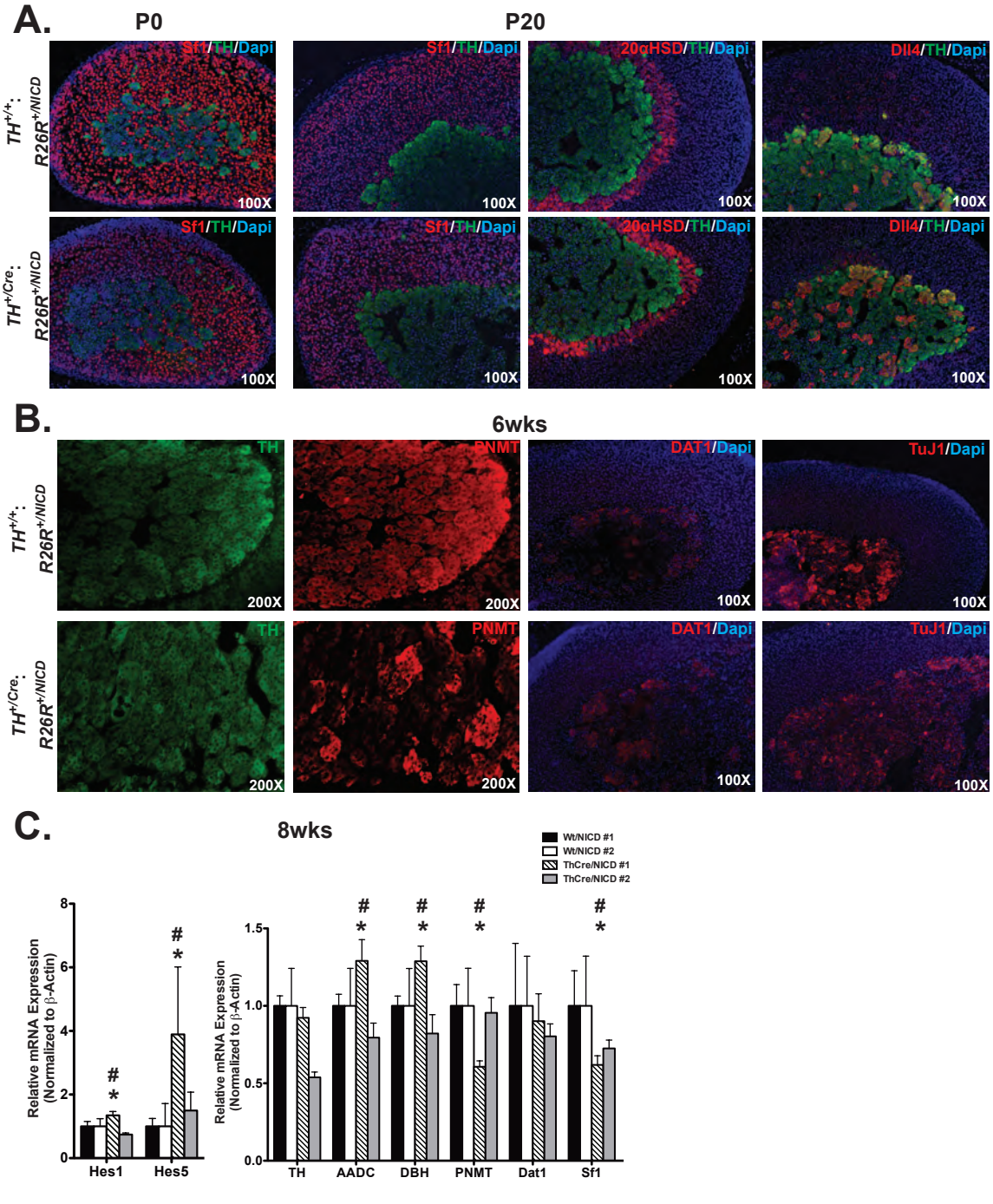

A.
P0
P20
TH+/+: R26R+/NICD
TH+/Cre: R26R+/NICD
Sf1/TH/Dapi
20αHSD/TH/Dapi
Dll4/TH/Dapi
100X
B.
6wks
TH
PNMT
DAT1/Dapi
TuJ1/Dapi
200X
100X
C.
8wks
Wt/NICD #1
Wt/NICD #2
ThCre/NICD #1
ThCre/NICD #2
Relative mRNA Expression (Normalized to β-Actin)
Hes1
Hes5
TH
AADC
DBH
PNMT
Dat1
Sf1

Figure 3.11: Inhibition of canonical Notch signaling in Sf1 positive cells has no effect on the adrenal glands of female mice. A. Histology of adrenals from *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ female mice between 18 and 50wks of age. **B.** Relative expression QPCR analysis (triplicate determinations) of mRNA from adrenals of 35wk *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$ (n=1), *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ (n=2), and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ (n=2) female mice. Immunofluorescence analysis of 35wk (**C.**) and 50wk (**D.**) *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ male mice for Cyp11b1 (red), TH (green), Sf1 (red) costained with TH (green) and β-catenin (red). Dapi is used as counterstain.

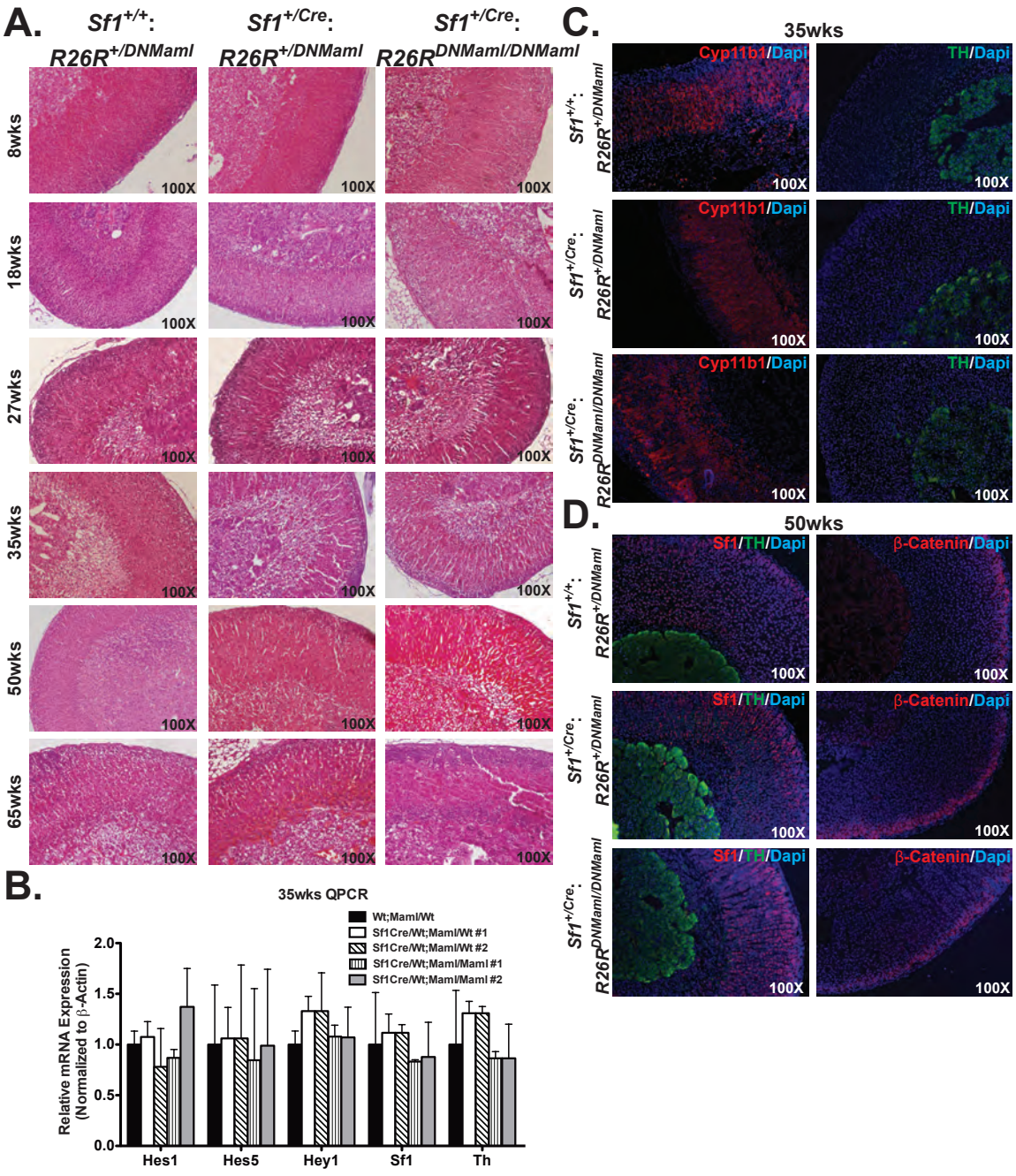

A.
Sf1+/+:
R26R+/DNMaml
Sf1+/Cre:
R26R+/DNMaml
Sf1+/Cre:
R26RDNMaml/DNMaml
8wks
18wks
27wks
35wks
50wks
65wks
100X
B.
35wks QPCR
Wt;Maml/Wt
Sf1Cre/Wt;Maml/Wt #1
Sf1Cre/Wt;Maml/Wt #2
Sf1Cre/Wt;Maml/Maml #1
Sf1Cre/Wt;Maml/Maml #2
Relative mRNA Expression
(Normalized to β-Actin)
2.0
1.5
1.0
0.5
0.0
Hes1
Hes5
Hey1
Sf1
Th
C.
35wks
Cyp11b1/Dapi
TH/Dapi
D.
50wks
Sf1/TH/Dapi
β-Catenin/Dapi

Figure 3.12: Inhibition of canonical Notch signaling in Sf1 positive cells has no effect on the adrenal glands of male mice. A. Histology of adrenals from *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ male mice between 18 and 50wks of age. **B.** Immunofluorescence analysis of adrenals from 50wk *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ male for Sf1 (red) costained with TH (green) and β-catenin (red). Dapi is used as counterstain.

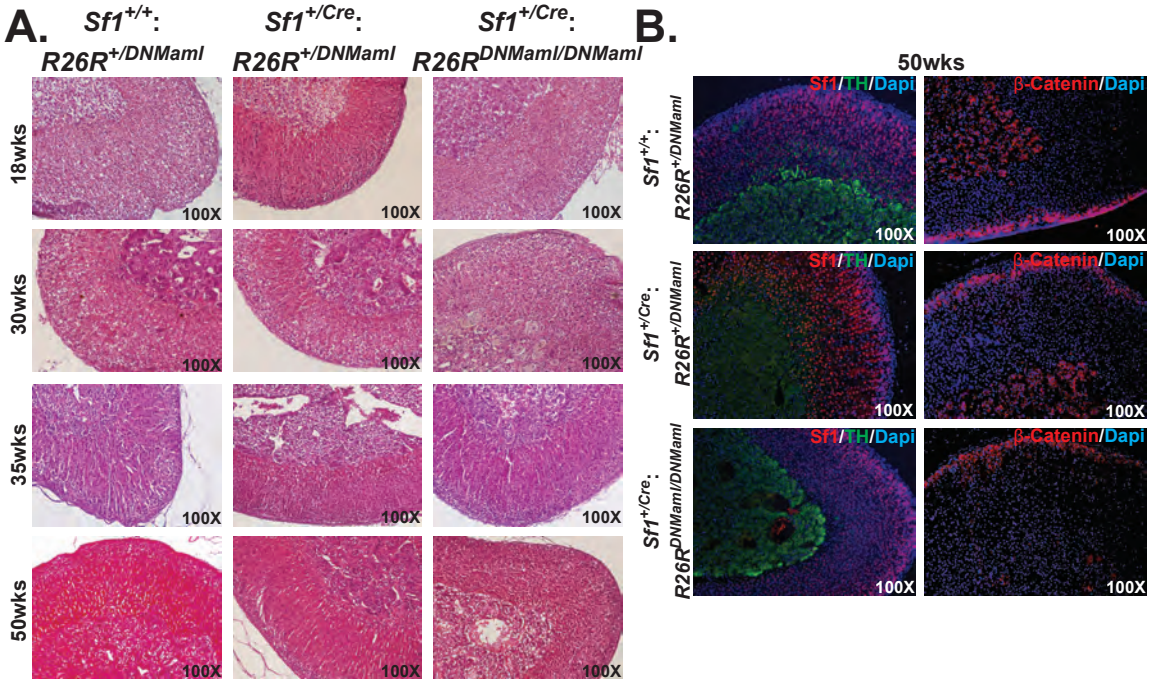

A.
Sf1+/+: R26R+/DNMaml
Sf1+/Cre: R26R+/DNMaml
Sf1+/Cre: R26RDNMaml/DNMaml
18wks
30wks
35wks
50wks
100X
B.
50wks
Sf1+/+: R26R+/DNMaml
Sf1+/Cre: R26R+/DNMaml
Sf1+/Cre: R26RDNMaml/DNMaml
Sf1/TH/Dapi
β-Catenin/Dapi
100X

Figure 3.13: Inhibition of canonical Notch signaling in Sf1 positive cells results in abnormalities in the ovary. **B.** Histology of ovaries from *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$, *Sf1*$^{+/Cre}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ female mice between 4 and 30wks of age. **C.** Immunohistochemistry for Sf1 in ovaries from 9 weeks old *Sf1*$^{+/+}$:*R26R*$^{+/DNMaml}$ and *Sf1*$^{+/Cre}$:*R26R*$^{DNMaml/DNMaml}$ female mice.

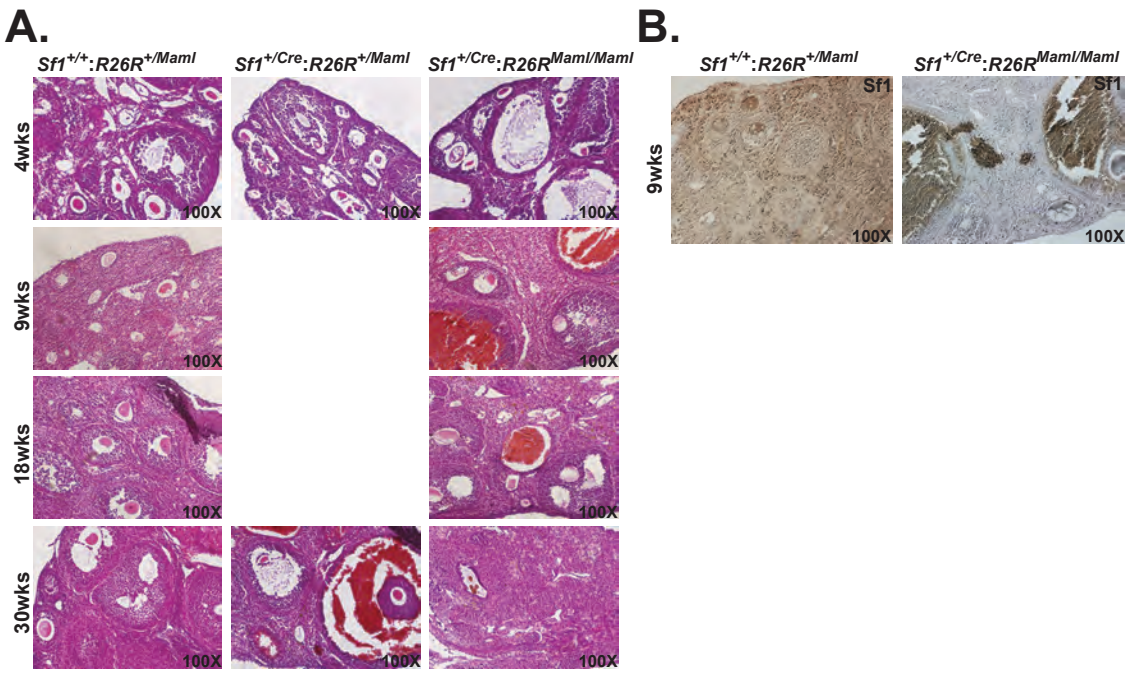

A.
Sf1+/+:R26R+/Maml
Sf1+/Cre:R26R+/Maml
Sf1+/Cre:R26RMaml/Maml
4wks
9wks
18wks
30wks
100X
B.
Sf1+/+:R26R+/Maml
Sf1+/Cre:R26RMaml/Maml
9wks
Sf1
100X

Figure 3.14: Overactivation of Notch signaling in Sf1 positive cells results in stochastic adrenal aplasia. A. The urogenital track of *Sf1*$^{+/+}$:*R26R*$^{+/NICD}$ and *Sf1*$^{+/Cre}$:*R26R*$^{+/NICD}$ P0 male and female mice, respectively. Adrenal glands (Ad) are indicated with yellow arrows. Gonads (Go) are indicated with red arrows. K: kidney, Bl: bladder. **B.** Zoomed in images of the left adrenal glands (Ad) superior to the kidney (K) in *Sf1*$^{+/+}$:*R26R*$^{+/NICD}$ and *Sf1*$^{+/Cre}$:*R26R*$^{+/NICD}$ P0 male mice. **C.** Immunofluoresence of *Sf1*$^{+/+}$:*R26R*$^{+/NICD}$ and *Sf1*$^{+/Cre}$:*R26R*$^{+/NICD}$ of P0 female (top panel) and male (bottom panel) mice for Sf1 (red), TH (green), and Dapi (blue).

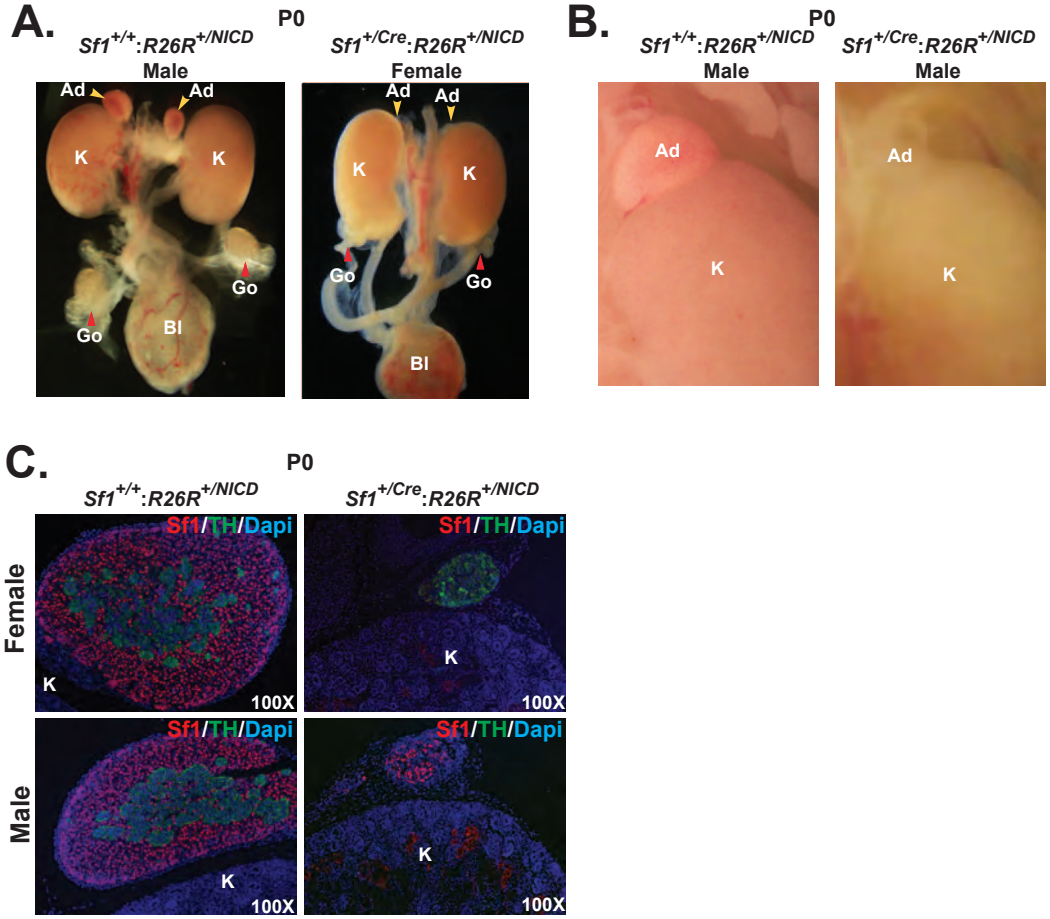

A.
P0
Sf1+/+:R26R+/NICD
Male
Sf1+/Cre:R26R+/NICD
Female
Ad
K
Go
Bl
B.
P0
Sf1+/+:R26R+/NICD
Male
Sf1+/Cre:R26R+/NICD
Male
Ad
K
C.
P0
Sf1+/+:R26R+/NICD
Sf1+/Cre:R26R+/NICD
Female
Male
Sf1/TH/Dapi
K
100X

Table 3.1: Genotyping Primers

Mouse Line	Primer #1	Primer #2	Primer #3
HesGFP	5'-gcacgacttcttcaagtccgccatgcc-3'	5'-gcggatcttgaagttcaccttgatgcc-3'	
Sf1Cre	5'-caatttactgaccgtacac-3'	5'-agctggcccaaatgttgctg-3'	n.a.
THCre	5'-gcggtctggcagtaaaaactatc-3'	5'-gtgaaacagcattgctgtcactt-3'	
Rosa Tomato/EGFP	5'-ctctgctgcctcctggcttct-3'	5'-cgaggcggatcacaagcaata-3'	5'-tcaatgggcgggggtcgtt-3'
Rosa DNMaml	5'-aaagtcgctctgagttgttat-3'	5'-gcgaagagtttgtcctcaacc-3'	5'-ggagcgggagaaatggatatg-3'
Rosa NICD	5'-taagctgcccagaagactc-3'	5'-gaaagaccgcgaagagtttg-3'	5'-aaagtcgctctgagttgttat-3'
SRY	5'-aagcgccccatgaatgcatt-3'	5'-cgatgaggctgatatttata-3'	n.a.

Table 3.2: Mouse QPCR primers

Primer Name	Forward Sequence	Reverse Sequence
Jag1	5'-gaggcgtcctctgaaaaaca-3'	5'-acccaagccactgttaagaca-3'
Jag2	5'-tcctcctgctgctttgtgat-3'	5'-tgtcaggcaggtcccttg-3'
Dll1	5'-acagaggggagaagatgtgc-3'	5'-ccctggcagacagattgg-3'
Dll3	5'-ctgcctgatggcctcgta-3'	5'-gctgctctctccaggtttca-3'
Dll4	5'-aggtgccacttcggttacac-3'	5'-gggagagcaaatggctgata-3'
Notch1	5'-aatcatgaggggtgtgaagc-3'	5'-ggatgctgactgcatggat-3'
Notch2	5'-ttacctaacacaacggcaca-3'	5'-ggcaatattctcccaagaagc-3'
Notch3	5'-agctgggtcctgaggtgat-3'	5'-agacagagccggttgtcaat-3'
Notch4	5'-ggacctgcttgcaaccttc-3'	5'-ctcacagagcctcccttcc-3'
Hes1	5'-acaccggacaaaccaaagac-3'	5'-cgcctcttctccatgatagg-3'
Hes5	5'-ccaaggagaaaaaccgactg-3'	5'-cttggagttgggctggtg-3'
Hey1	5'-catgaagagagctcacccaga-3'	5'-cgccgaactcaagtttcc-3'
TH	5'-cccaagggcttcagaagag-3'	5'-gggcatcctcgatgagact-3'
AADC	5'-ctttgactgctctgccatgt-3'	5'-tccatattaaaggctccggtta-3'
DBH	5'-atctccatgcattgcaacaa-3'	5'-aggctgcagattccactcac-3'
PNMT	5'-cagcatgcctgcctcatt-3'	5'-aggcaggactcgcttcac-3'
COMT	5'-ccgctaccttccagacacac-3'	5'-gttcccgggacaatgaca-3'
DAT1	5'-ccttatcctttgtcgcagaga-3'	5'-aaaggcagggatgagcttg-3'
NAT1	5'-gccgtcctgttcttcttgat-3'	5'-cttccatgcctcccattg-3'
ChrA	5'-cgatccagaaagatgatggtc-3'	5'-cggaagcctctgtctttcc-3'
SF1	5'-tccagtacggcaaggaagac-3'	5'-ctgtgctcagctccacctc-3'
Beta-Actin	5'-ctaaggccaaccgtgaaag-3'	5'-accagaggcatacagggaca-3'

Table 3.3: Antibodies

Antibody Name	Species	Company	Catalog #	Notes
Jagged1 (C-20)	Goat	Santa Cruz	Sc-6011	
JAG1	Rabbit	Abcam	Ab7771	
Dll4	Rabbit	Abcam	Ab7280	
Notch1 (C-20)	Goat	Santa Cruz	Sc-6014	
Notch2 (25-255)	Rabbit	Santa Cruz	Sc-5545	
Dlk1	Rat	N.A.	N.A.	A generous gift from Charlotte Harken Jensen
Sf1	Rabbit	N.A.	N.A.	A custom polyclonal antibody
TH	Mouse	Millipore	MAB318	
PNMT	Rabbit	Milipore	AB110	
DAT1	Rat	Millipore	MAB369	
TuJ1	Mouse	Covance	MMS-435P	
Cyp11b1	Rat	N.A.	N.A.	A generous gift from Celso Gomez-Sanchez
20alphaHSD	Rabbit	N.A.	N.A.	A generous gift from Yacob Weinstein
Beta-Catenin	Mouse	BD Biosciences	610154	
GFP	Rabbit	Invitrogen	A11122	
Beta-Actin	Mouse	Sigma	A-5411	
Anti-Goat IRDye	Donkey	LICOR	926-32214	
Anti-Mouse IRDye	Goat	LICOR	926-32220	
Anti-Rabbit IgG HRP	Goat	Pierce	31462	
Anti-Rat Dylight 549	Goat	Jackson ImmunoResearch	112-505-044	
Anti-Rabbit Dylight 549	Goat	Jackson ImmunoResearch	111-506-045	
Anti-Goat Dylight 549	Rabbit	Jackson ImmunoResearch	305-505-045	
Anti-Mouse Dylight 549	Goat	Jackson ImmunoResearch	115-485-174	
Anti-Mouse Dylight 488	Goat	Jackson ImmunoResearch	115-095-008	

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Chapter 4: Conclusions and Future Directions

The proper receipt and transmission of molecular signals is essential for cellular function during development and homeostasis. Improper cell-cell communication can lead to abnormalities in the development of organs [1], failure in maintenance of organ homeostasis [2], or even cancer [3]. The evolutionarily conserved Notch pathway has been implicated in many of these processes in a variety of tissue types [4]. Notch signaling occurs through interactions between transmembrane ligands and receptors and culminates in the release of the active signaling molecule, the cleaved Notch intracellular domain (NICD) [5]. Therefore, canonical Notch signaling requires direct cell-cell contact. However, unlike most signaling pathways, no signal amplification occurs in Notch signaling because each Notch receptor can only generate a single NICD molecule [6]. These unique aspects of Notch pathway activation allow for incredible fine-tuning in the type of signals that Notch can receive and transmit. It is not surprising that Notch signaling has been implicated in a host a developmental disorders as well as cancer [1, 4]. However, Notch signaling has never been considered in the adrenal glands, endocrine organs responsible for coordinating the mammalian stress response. Understanding the role of Notch signaling in the adrenal glands may reveals insights into normal and pathogenic adrenal biology.

In Chapter 2 of this thesis, we interrogated the role of Notch signaling in adrenocortical cancer. Adrenocortical tumors are common with benign adrenocortical adenomas (ACA) present in about 4-7% of the population while adrenocortical carcinomas (ACC) present at a much lower frequency (about 1-2 cases/million/year) but are highly aggressive, deadly neoplasms [7, 8]. We have identified an upregulation of the Notch ligand Jagged1 (JAG1) in ACC compared to ACA and normal adrenals. An upregulation of the Notch receptors Notch2 and Notch3 was also observed and Notch3 expression correlated with JAG1 expression. Notch target genes Hes1 and Hey1 were also upregulated in ACC. Taken together these data suggest an overactivation of canonical Notch signaling is occurring in ACC.

Indeed, we have shown that Jag1 mediates a non-cell autonomous effect on cell proliferation in the Y1 mouse ACC cell line. Knockdown of Jag1 in Y1 cells resulted in a non-cell autonomous reduction in cell proliferation while inhibition of all downstream canonical Notch signaling phenocopies the Jag1 knockdown experiments. Furthermore, JAG1 expression was found to correlate with late stage and high grade human ACC, as well as markers of proliferation, TOP2A and KI67. These data support a role of Jag1-dependent Notch receptor activation in exacerbating the ACC phenotype by augmenting cellular proliferation of ACC cells.

The observation that JAG1 expression correlates with late stage ACC suggest that JAG1 may not be involved in initiation of ACC tumorigenesis but may function in progression to a more aggressive ACC phenotype. Indeed, a

multitude of factors and signaling pathways have been implicated in ACC pathogenesis [9]. Constitutive activation of β-catenin, the downstream effector of Wnt signaling, has been shown to cause adrenocortical hyperplasia and microscopic adenomas [10, 11]. Insulin-like growth 2 (IGF2), a ligand of the IGF signaling pathway, is consistently identified at the most upregulated gene in ACC compared to ACA and normal adrenals [12, 13]. We have recently published a study in which these two factors have been found to synergize in the development of adrenocortical tumors in the laboratory mouse [10]. However, a lack of *bona fide* ACC was reported which suggests that overactivation of Wnt and IGF pathways are not sufficient to drive adrenocortical carcinogenesis.

The Notch pathway may be involved in the progression to ACC. The status of the Notch signaling pathway has not been interrogated in the tumors that developed in the Wnt/IGF study [10]. A comprehensive analysis of Jag1 expression in these tumors may reveal an upregulation of Jag1 in the more histopathologically severe tumors. This observation would confirm that Jag1 upregulation concomitant with overactivation of the Wnt and IGF pathways is required for progression Furthermore, other adrenal tumors models have been reported but Jag1 expression has not been considered in any of these models [11, 14]. Future analyses may entail interrogation of the status of Jag1 expression in these mouse adrenal tumor models and consideration if the level of Jag1 expression correlates with tumor aggressiveness and/or proliferation.

In Chapter 3 of this thesis, we examined the function of Notch signaling in normal adrenocortical cells during development. Jag1 expression can be

detected in the subcapsular region of the adrenal cortex. This zone has previously been shown to be involved in adrenocortical homeostasis [2, 15]. However, inhibition of canonical Notch signaling in Sf1+ cells had no effect on the development or maintenance of the adrenal cortex. These data contrast with the role of Notch signaling in ACC. However, the molecular profile of a cancer cell is often drastically different than the molecular profile of the normal cell from which the cancer is derived [12]. The function of a pathway in cancer does not predict the same function of that pathway in the normal cell. Indeed, our analysis of Notch in adrenocortical function appears to confirm this supposition.

Furthermore, overactivation of Notch signaling in Sf1+ cells resulted in adrenal aplasia. These results are difficult to interpret due to the lack of endogenous Notch activity in normal Sf1+ adrenocortical cells. The *Sf1*$^{+/Cre}$ mouse line employed in these studies is also active as early as E10.5, which suggest the adrenal failure can occur at anytime between E10.5 and E18.5 [16]. A future study that is far more interesting and biologically relevant would be to overactivate canonical Notch signaling and/or overexpress Jag1 in Sf1+ cells of the adult adrenal cortex concurrent with stabilization of β-catenin and/or the IGF pathway. These experiments would help to elucidate whether Notch pathway overactivation can exacerbate adrenocortical tumorigenesis in a molecular environment that is already predisposed to the development of benign adrenoocortical tumors. It is plausible that overexpression of Jag1 and/or constitutive stabilization of Notch signaling in the context of overactivation Wnt

and/or IGF signaling may results in progression to *bona fide* ACC in the laboratory mouse.

Alternatively, in Chapter 3 of this thesis, we instead identified that Notch signaling is active in chromaffin cells of the adrenal medulla. The adrenal medulla is of distinct ontogeny and function from the adrenal cortex; it is neural crest-derived and secretes the catecholamine hormones epinephrine and norepinephrine whereas the cortex is mesoderm-derived and secretes steroid hormones. We identified expression of the canonical Notch target genes Hes1 and Hes5, ligands Jag1 and Dll4, noncanonica ligand Dlk1, and receptors Notch1 and Notch2 in adult chromaffin cells. Furthermore, Hes1 and Hes5 expression was identified in putative chromaffin progenitors pre- and post- migration into the adrenal primordia during medullary development. When canonical Notch signaling was inhibited in tyrosine hydroxylase (TH)+ cells (TH is the rate limiting enzyme in catecholamine synthesis and is expressed in every adrenal medullary chromaffin cell [17]), we observed no changes in the overall histology of chromaffin cells but identified an upregulation in TH mRNA and protein. The lack of a more prominent phenotype may be a consequence of the mosaic expression of the $TH^{+/Cre}$ line employed in this study. A sufficient number of chromaffin progenitors may avoid Notch inhibition and develop to mature chromaffin cells. Furthermore, during development, a distinct population of cells that are Hes1+/TH- was detected in the vicinity of the adrenal primordia. Other studies have suggested that some chromaffin progenitors may lack TH expression prior to invasion of the adrenal primordia [18]. Therefore, we may not be targeting

bona fide chromaffin progenitors cells and thus not affecting development of the adrenal medulla.

Nevertheless, the $TH^{+/Cre}$ line is still active in some TH+ cells of the adult adrenal medulla and therefore, Notch inhibition is occurring in a subpopulation of adult chromaffin cells in this study. The observation that TH mRNA and protein are upregulated suggests a Notch-dependent inhibition of TH expression may be occurring in adult chromaffin cells. However, the broad expression of Hes1 and the Notch ligands throughout the TH+ adrenal medulla argues against the complete suppression of TH expression by Notch signaling. Instead, Notch may serve to fine-tune the amount of TH that is expressed in order for the medulla to properly respond to different stressors. Future experiments may entail various stress paradigms in Notch inhibition mice and measurements of changes of catecholamines and/or their metabolites that may occur in this model under stressed and unstressed conditions.

In contrast, when we overactivated Notch signaling in TH+ cells, we observed no changes in the overall histology of chromaffin cells except for a reduction in phenylethanolamine-N-methyltransferase (PNMT) expression, an enzyme required for synthesis of epinephrine and marker of adrenergic chromaffin cells [17]. These data suggest that overactivation of Notch signaling may reduce the population of adrenergic chromaffin cells. However, only a few animals have been analyzed and a much larger sample size is needed to confirm this intriguing observation. Furthermore, since the loss of PNMT expression was observed in 6wk old animals but was not examined in neonatal animals, a more

obvious affect on PNMT expression may be observed in aged animals. Measurement of circulating catecholamine hormones and/or their metabolites would reveal if there is differences in the amount of epinephrine being secreting from the adrenal medulla because the medulla is the primary site if epinephrine synthesis and secretion [17, 19].

Our Notch inhibibition and overactivation studies reveal a differential requirement of Notch signaling in regulating the expression of catecholamine-synthesizing enzymes; reduction in Notch signaling results in TH upregulation while overactivation results in PNMT downregulation. These observations seem to contradict our observation of Notch signaling throughout the adrenal medulla concomitant with TH and PNMT expression. These data suggest that Notch in the medulla may be not “all or none” but may be specific to each chromaffin cell-cell contact. That is to say, too much Notch signaling may reduce PNMT gene expression but the “right” amount may promote it. The same type of argument could be made for the regulation of TH expression; not enough results in upregulation were just the “right” amount maintains steady-state levels. Indeed, the different expression patterns of Hes1 versus Hes5 and Dll4 compared to Jag1 seem to suggest that differential Notch ligand-receptor signaling may be occurring in subpopulations of chromaffin cells. However, the effect of Notch on catecholamine-synthesizing enzymes may also be indirect, mitigated through other pathways and/or transcription factors.

Furthermore, the noncanonical Notch ligand Dlk1, which we detect throughout the medulla (as has been reported previously [20]), may play a role in

the balance of Notch signaling between chromaffin cells. Dlk1 has been suggested to inhibit Notch signaling in a cell-autonomous manner but this not been experimentally confirmed as a consequence of direct Dlk1 binding to Notch receptors [21]. In our system, Dlk1 may indeed inhibit some Notch signaling in chromaffin cells in order to allow for the proper dosage of the Notch signal required by an individual chromaffin cells under different conditions. Analysis of Dlk1 knockout mice may reveal insights into this putative role.

Finally, the Notch overactivation studies may have implications in pheochromocytoma (PCC), a catecholamine-secreting neoplasm derived from the adrenal medulla. Gene expression profiling of PCC identified an upregulation of Jag1 and Notch3 in both sporadic and hereditary norepinephrine-secreting PCC compared to hereditary and sporadic epinephrine-secreting PCC [22]. These data supplement our observation that Notch overactivation reduces PNMT expression and suggests that Notch overactivation may promote a noradrenergic-like phenotype. Additional studies on a larger cohort of PCC would be necessary to confirm this observation. It is possible that overactivation of Notch signaling in TH+ cells may even yield PCC in aged animals. In the hereditary cancer disorder, von-Hippel Lindau syndrome, an autosomal dominant disorder in which the *VHL* tumor suppressor gene is mutated, PCC develop that are norepinephrine-secreting [23]. To interrogate the function of Notch signaling in norepinephrine-secreting PCC, one possible avenue of investigation would be overactivaiton of Notch signaling in chromaffin cells that are deficient for the *VHL*

gene. It is possible that norepinephrine-secreting PCC may develop under these conditions.

In this thesis we have characterized Notch signaling in adrenocortical cancer and in the normal adrenal medulla. These studies illustrate the highly context-dependent role of Notch in various systems. While a great deal of work is needed to further characterize the precise role of Notch in the medulla, our data support a role of Notch signaling in modulation of catecholamine production from chromaffin cells. Furthermore, the identification of an upregulation of JAG1 in ACC is intriguing but the mechanism of JAG1 upregulation and the complement of genes that Notch signaling regulates in ACC still need to be elucidated. Targeting Jag1-mediated Notch signaling in late stage ACC may represent a novel therapeutic option in the treatment of ACC. This plausibility of this idea is highlighted by the existence of a variety of pharmacological methods to inhibit Notch signaling in a clinical setting [24]. Further characterization of Notch signaling in ACC may open avenues of research that culminate in improved treatment for this dismal disease.

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