Investigating the Mechanism of a Multi-State Model of WNT Signaling

by

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ABSTRACT

The WNT signaling pathway plays numerous roles in development and maintenance of adult homeostasis. In concordance with its numerous roles, dysfunction of WNT signaling leads to a variety of human diseases ranging from developmental disorders to cancer. WNT signaling is composed of a family of 19 WNT soluble secreted glycoproteins, which are evolutionarily conserved across all phyla of the animal kingdom. WNT ligands interact most commonly with a family of receptors known as frizzled (FZ) receptors, composed of 10 independent genes. Specific interactions between WNT proteins and FZ receptors are not well characterized and are known to be promiscuous, Traditionally canonical WNT signaling is described as a binary system in which WNT signaling is either off or on. In the 'off' state, in the absence of a WNT ligand, cytoplasmic β-catenin is continuously degraded by the action of the APC/Axin/GSK-3β destruction complex. In the 'on' state, when WNT binds to its Frizzled (Fz) receptor and LRP coreceptor, this protein destruction complex is disrupted, allowing β-catenin to translocate into the nucleus where it interacts with the DNA-bound T cell factor/lymphoid factor (TCF/LEF) family of proteins to regulate target gene expression. However in a variety of systems in development and disease canonical WNT signaling acts in a gradient fashion, suggesting more complex regulation of β-catenin transcriptional activity. As such, the traditional 'binary' view of WNT signaling does not clearly explain how this graded signal is transmitted intracellularly to control concentration-dependent changes in gene expression and cell identity. I have developed an in vitro human pluripotent stem cell (hPSC)-based model that recapitulates the same in vivo developmental effects of the WNT signaling gradient on the anterior-posterior (A/P) patterning of the neural tube observed during early development. Using RNA-seq and ChIP-seq I have characterized β-catenin binding at different levels of WNT signaling and identified different classes of β-catenin peaks that bind cis-regulatory elements to influence neural cell fate. This work expands the traditional binary view of canonical WNT signaling and illuminates WNT/β-catenin activity in other developmental and diseased contexts.

DEDICATION

To my parents Matt and Ellen, my brother Zach, and my sister Hannah, for their unconditional support and unwavering belief in me. I love you all.

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

PREFACE

Chapter 2 presented in this PhD dissertation document, have been previously published as described below:

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CHAPTER 1

INTRODUCTION

1.1. Stem Cells and Pluripotency

Stem cells are defined quite simply as cells that have the capacity to indefinitely self renew and differentiate into more terminal cell types (Ding & Schultz, 2004). This broad definition encompasses two overarching categories of stem cells, pluripotent stem cells and adult stem cells. Adult stem cells are rare resident stem cells that reside in postnatal stem cell niches, maintaining populations of adult cells in organisms. While pluripotent stem cells have the capacity to be differentiate into almost any cell type in the body, and are typically found in embryos.

1.1.1. Discovery of embryonic stem cells

The first isolation of pluripotent stem cells was the embryonic stem cell (ESC), first isolated in mice in 1981 by Evans and Kauffman (Evans & Kaufman, 1981). This breakthrough discovery required careful optimization the isolation of mouse blastocysts with identification of passaging conditions required to maintain them. Their work paid off and yielded cell lines with a normal karyotype capable of being serially passaged and differentiating into all three germ layers in teratocarcinomas (Evans & Kaufman, 1981). Gail Martin subsequently published a method for isolating mouse embryonic stem cells (mESCs) from the inner cell mass (ICM) of embryos instead of culture of whole blastocysts that was employed by Evans and Kauffman (G. R. Martin, 1981). Seventeen years passed between the initial isolation of mESCs and human embryonic stem cells (hESCs) by Jaime Thompson in 1998 using an ICM isolation technique (Thomson et al., 1998).

The breakthrough discovery of stem cells enabled the study of basic science in many ways including developmental biology, disease modeling, as well as more applied therapeutic interventions such as high throughput drug screening, and cell replacement therapies. The isolation of murine embryonic stem cells along with the discovery of homologous recombination opened up the potential to use these cells to study development in a groundbreaking new way enabling the generation of reporter lines, genetic knockouts, and conditional genetic knockouts in

mice and cell culture platforms, which are powerful tools to assess the specific effects of genes on aspects of development (Babinet & Cohen-Tannoudji, 2001; Limaye, Hall, & Kulkarni, 2009; Vasquez, Marburger, Intody, & Wilson, 2001). ESCs provided the ability to create chimeras in edited mESCs, able to contribute to the generation of every tissue of the new animal. By analyzing the phenotype of knockout mice it was possible to deduce the function of a gene. This process, although not always perfect allowed the generation of a framework for how genes affect development and different phenotypes. Some genes that are critical for development, when knocked out, lead to an embryonic lethal phenotype, severely impairing study into their action. This problem can be overcome by several approaches, including conditional knockouts using a cre/loxp system or by introducing mutations within a gene that are less severe than an gene knockout (Sakamoto, Gurumurthy, & Wagner, 2014). The discovery of stem cells has made a tremendous impact on the field of developmental biology.

Thompson et. al. immediately noted the enormous therapeutic potential of hESCs as a limitless source of differentiated cell types for cell replacement therapies for diseases such as Diabetes Mellitus, Parkinson's disease, or spinal cord injury (Thomson et al., 1998). Cell replacement therapy could be thought of as the holy grail of stem cell research, which many scientists across the world still labor towards. When hESCs were first derived protocols for therapeutic relevant differentiations were developed such as for neurons or cardiomyocytes, but these needed to be dramatically improved and scaled for any attempt at human therapies. However since then numerous studies have documented the ability to generate almost any cell type with high purity including cortical neurons (Shi, Kirwan, Smith, Robinson, & Livesey, 2012), cholinergic neurons (Maury et al., 2015), dopaminergic neurons (Nolbrant, Heuer, Parmar, & Kirkeby, 2017), oligodendrocytes (Douvaras & Fossati, 2015), astrocytes (Shaltouki, Peng, Liu, Rao, & Zeng, 2013), cardiomyocytes (Burridge et al., 2014), hepatocytes (Roelandt, Vanhove, & Verfaillie, 2013), kidney glomerular podocytes (Musah, Dimitrakakis, Camacho, Church, & Ingber, 2018), pancreatic β-cells (Rosado-Olivieri, Anderson, Kenty, & Melton, 2019), and many more. A problem that still plagues the field however is the generation of mature functional cell types using directed differentiation protocols. Many labs are working on techniques to mature these cells in

vitro to enable their use as cell therapeutics. However ethical challenges and the labor-intensive nature of hESC derivation provided major roadblocks to their use in the clinic (Hyun, 2011).

1.1.2. Discovery of induced pluripotent stem cells

Groundbreaking work by Shinya Yamanaka first in mouse then in human fibroblasts demonstrated that viral overexpression of four transcription factors Oct4, Sox2, c-Myc, and Klf4 yielded ES like cells, dubbed induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Takahashi & Yamanaka, 2006). This incredible achievement was in part possible through pioneering somatic cell nuclear transfer experiments by John Gurdon, which demonstrated that DNA is conserved throughout differentiation and the differentiated cell state is reversible through factors present in oocytes (De Robertis & Gurdon, 1977; Gurdon, 1962; Gurdon & Uehlinger, 1966; Laskey & Gurdon, 1970; Wilmut, Schnieke, McWhir, Kind, & Campbell, 1997). Together these two scientists, Shinya Yamanaka and John Gurdon, shared the 2012 Nobel Prize in physiology or medicine for the discovery that mature cells can be reprogrammed to become pluripotent (Jaenisch, 2012). This discovery forever revolutionized the stem cell field enabling studies into the nature of pluripotency, the unimpeded derivation of patient specific stem cell lines, and paved the way for clinical implementation of stem cell therapies.

Initial retroviral based methods used to overexpress the Yamanaka factors subjected cells to insertional mutagenesis, which could lead to deleterious insertions akin to initial retroviral attempts at gene therapy (Hacein-Bey-Abina et al., 2003; McCormack & Rabbitts, 2004). To improve the safety of potential therapeutic applications of this technology much effort has been dedicated to developing non incorporating, or footprint free, methods to generate iPSCs. There are a range of methods available to generate iPSCs including RNA virus (sendai) (Fusaki, Ban, Nishiyama, Saeki, & Hasegawa, 2009), adenovirus (Stadtfeld, Nagaya, Utikal, Weir, & Hochedlinger, 2008), episomal (Okita et al., 2011; Okita, Nakagawa, Hyenjong, Ichisaka, & Yamanaka, 2008; Su et al., 2013), mRNA (Warren et al., 2010), protein (D. Kim et al., 2009; Zhou et al., 2009), and small molecule based (Hou et al., 2013). Interestingly, although derivation of iPSCs is now widely adopted and commonplace the efficiency remains very low (~1%) suggesting that this process is still not completely understood and could be dependent on some

unknown stochastic events (Yamanaka, 2012). Future work could uncover these dependencies and allow high efficiency generation of high quality iPSCs. With the advent of two competing pluripotent stem cells important questions were raised about the differences between these two cell types. Initial work demonstrated both transcriptomic and epigenomic differences between ESCs and iPSCs (Chin et al., 2009; Deng et al., 2009; Ghosh et al., 2010; K. Kim et al., 2011; Lister et al., 2011; Marchetto et al., 2009; Ohi et al., 2011). However studies that compared larger numbers of lines found it difficult to detect differences suggesting common mechanisms of pluripotency between the two (Bock et al., 2011; Guenther et al., 2010; Newman & Cooper, 2010). In summary iPSC quality is more dependent on technical aspects of derivation and that comprehensive characterization of lines used in research and potential therapies will be required before use (Yamanaka, 2012).

1.1.3. Utility of stem cells

Stem cells enable the study of basic science in the form developmental biology and disease modeling as well as enabling therapies to advance human health through the use of high throughput drug screening and cell therapeutics. Model organisms can be studied and modified extensively to provide valuable contributions to our understanding of development however the identical studies would not be ethically responsible to carry out in human embryos. Human pluripotent stem cells have dramatically advanced our understanding of human development by enabling rigorous examination of the events in early human development (Aach, Lunshof, Iyer, & Church, 2017; Warmflash, Sorre, Etoc, Siggia, & Brivanlou, 2014). Additionally iPSCs have facilitated the generation of patient specific lines which can be probed for differences in development or in terminally differentiated cells and compared to healthy lines to understand previously uncharacterized aspects of diseases (Y. Wang et al., 2014). This approach is exemplified in the study of neurodegenerative diseases (LaMarca, Powell, Akbarian, & Brennand, 2018; Meyer et al., 2019; Ross & Akimov, 2014; Rowland, Hooper, & Kellett, 2018; Schmid et al., 2019; Soldner et al., 2011). Patient specific lines not only allow mechanistic insight into disease progression but are being used as platforms to perform high content drug screens to identify pharmaceutical interventions for patients suffering from a variety of diseases (Ebert, Liang, & Wu,

2012; Mercola, Colas, & Willems, 2013; Xu & Zhong, 2013). Finally, many diseases are characterized by death or dysfunction in just a single cell type such as Parkinson's disease, Diabetes Mellitus, spinal cord injury, or macular degeneration. These diseases offer the tantalizing hope of using pluripotent stem cells as a raw material to provide cells for replacement therapies.

Stem cell technology is still in its infancy but it has rapidly expanded and developed into its own specialized field. Stem cells are used for a variety of purposes outlined above including developmental biology, disease modeling, and cell replacement therapies. The work described here used stem as a developmental biology platform to probe the fundamental mechanisms of WNT signaling.

1.2. WNT Signaling

The first WNT protein was discovered in 1976 in drosophila as a mutant allele that led to the loss of wing tissue. At the time it was dubbed wingless (wg) due to the observed phenotype and hypothesized function (Sharma & Chopra, 1976). Several years later an independent line of investigation by Roel Nusse and Harold Varmus, utilizing an approach to search for proto-oncogenes near mouse mammary tumor virus (MMTV) insertion sites, led to the discovery of a gene called int1. For many years the function of this protein remained elusive, as it was very difficult to isolate (Roel Nusse & Varmus, 2012). Eventual isolation of the drosophila int1 revealed that that wg and int1 were orthologs due to sequence homology (Rijsewijk et al., 1987). Subsequently the int nomenclature proved insufficient for classifying genes, since MMTV integration sites do not group genes together by function, and a large family of genes related to int1 had been discovered (Gavin, McMahon, & McMahon, 1990). Thus the field of WNT signaling was born of the amalgam of the original wg and int1 genes, which proved to be orthologues (R Nusse et al., 1991).

WNTs compose a large family comprising an evolutionarily conserved signaling pathway that constitute a secreted glycoprotein which regulate many cell processes including proliferation, differentiation, cell polarity, primary axis formation, cell cycle, and developmental programs of multiple organs (Estarás, Benner, & Jones, 2015a; Habas & Dawid, 2005; Komiya & Habas,

2008; Loh, van Amerongen, & Nusse, 2016). As WNT signaling controls such a diverse set of processes, mutations in the WNT pathway lead to many different types of diseases including multiple cancers (Clevers & Nusse, 2012a).

1.2.1. WNT family of proteins

The WNT family consists of 19 different WNT proteins. WNT proteins share a common structure of a stretch of 22 cysteine residues, which form disulfide bridges conferring its globular structure. Additionally there is a sequence of 20 hydrophobic amino acids, a hallmark of secreted proteins (K. Willert & Nusse, 2012). The structures for three WNT proteins have been solved in xenopus, drosophila, and most recently human providing insight into the function of this elusive group of proteins. The recent solved structure of human WNT3 bound to mouse Fzd 8 is an important step forward for the field since Wnt3 is a known canonical activator of WNT signaling. This structure revealed similarities to the previously solved xenopus structure, which was also conjugated to Fzd8. These structures revealed that WNT proteins are shaped reminiscent of a hand, consisting of two structural subdomains the N-terminal domain (NTD) C-terminal domain (CTD) with a variable linker domain tying the two together. Palmoteic acid modification is invariably located at serine residue (187), which is essential for binding the cysteine rich domain (CRD) of Fzd receptors (D.H. Kim et al., 2012). In WNT3 the linker domain was demonstrated to be essential for LRP6 coreceptor binding, a function hypothesized by earlier studies (Chu et al., 2013; Hirai, Matoba, Mihara, Arimori, & Takagi, 2019). Somewhat controversially a homodimeration between WNT3-Fzd8 WNT3-Fzd8 complexes was observed in crystals that has been previously hypothesized to be impossible (Hirai et al., 2019; Nile, Mukund, Stanger, Wang, & Hannoush, 2017). Future work may elucidate distinct signaling efficiencies that may be associated with different ratios of WNT-Fzd complexes (Hirai et al., 2019).

WNT secretion is a complex process, involving covalent modification with a lipid palmitoleic (PA) acid group by the acyl transferase porcupine (PRCN) in the ER, conferring WNTs renowned hydrophobicity. This property halted the purification of bioactive WNT for nearly two decades after its initial discovery, and still complicates isolation of additional WNTs (K. Willert et al., 2003). Additionally this modification complicates use of bioactive isolated WNTs, since

detergents are required to maintain its solubility (D. Brafman & Willert, 2017; K. Willert et al., 2003). This modification serves to restrict WNT signalings' range to short distances. After covalent addition of palmitoleic acid WNTs associate with Wls (Gpr177/Evi) a transmembrane protein required to shuttle WNTs to the cell surface (16678096,16678095). The function of PRCN and Wls to modify and secrete WNTs are essential to WNT maturation and release as indicated by the fact that mutations or inhibition of these proteins results in phenotypes similar to WNT3 knockouts (Barrow et al., 2007; Biechele, Cox, & Rossant, 2011; Fu, Jiang, Mirando, Yu, & Hsu, 2009; C. Liu et al., 1999).

1.2.2. WNTs as morphogen gradients

Establishment and interpretation of morphogen gradients are an essential principle of developing organisms (Gurdon & Bourillot, 2001). The original concept concerning interpretation of morphogen gradients was pioneered by Lewis Wolpert, he hypothesized that specific thresholds of morphogens determine cell identity, an idea that has stood the test of time (Wolpert, 1969). There are multiple examples of Wnt morphogen gradients in development including the drosophila wing (Zecca, Basler, & Struhl, 1996), anteroposterior neural patterning (Kiecker & Niehrs, 2001; Moya, Cutts, Gaasterland, Willert, & Brafman, 2014), mouse intestinal crypts (Farin et al., 2016), and hematopoietic stem cells (Tiago C. Luis et al., 2011). How these gradients are established is an area under active fruitful investigation, whether the mechanism is through long or short distances is a contentious topic. Due to their known highly hydrophobic nature, Wnt proteins are mainly hypothesized to act through short distance mechanisms (K. Willert et al., 2003). Strong evidence exists to support this idea in that tethered WNT in drosophila mutants develop normally (Alexandre, Baena-Lopez, & Vincent, 2013). Additional evidence that the prevalent mechanism of WNT signaling is through short range gradients comes from visualization of the Wnt3 gradient in intestinal cells through the use of intestinal organoids in the Clevers lab (Farin et al., 2016). This work demonstrates that WNT ligands are transferred to neighboring Lgr5+ cells and a gradient is established through cell division, which dilutes the signal. Although it seems short range action is the prevalent form of signaling several long range mechanisms exist which may have supplemental effects on establishing gradients of WNT signaling including

chaperone proteins (Mihara et al., 2016), actin based membrane protrusions (H. Huang & Kornberg, 2015; Stanganello & Scholpp, 2016) and extracellular particles (Beckett et al., 2013; Q. Chen, Takada, Noda, Kobayashi, & Takada, 2016; J. C. Gross, Chaudhary, Bartscherer, & Boutros, 2012; T. Harada et al., 2017). Future work may elucidate that alternative mechanisms of WNT release may play important context specific roles in development and disease.

1.2.3. Frizzled receptors

WNT proteins bind frizzled (FZD) receptors on the cell membrane, a large group of proteins consisting of 10 genes identified in organisms ranging from sponges to humans (H.-C. Huang & Klein, 2004). Frizzled receptors have seven transmembrane domains and are composed of a signal sequence, an extracellular cysteine rich domain (CRD), a hydrophobic transmembrane domain, and a c-terminal KTXXXW motif essential for canonical WNT signaling through disheveled (DVL) (Bourdelas et al., 2015; Umbhauer et al., 2000). Specific WNT-FZD interactions are known to be somewhat promiscuous although exact relationships still remain unknown, in part due to difficulties in isolating a majority of known WNT proteins (K. Willert et al., 2003). R-spondins are secreted proteins that are able to enhance levels of WNT signaling by modulating availability of FZD receptors but unable to activate WNT signaling alone (de Lau, Snel, & Clevers, 2012). Future work characterizing exact relationships between specific WNTs and FZD receptors could further help elucidate functions and mechanisms of WNT signaling.

1.2.4. Canonical WNT signaling

WNT signaling is classically divided into two categories, canonical and non-canonical. Non-canonical WNT signaling is typically not as well understood as canonical signaling; its activity is characterized by non β-catenin activity and is composed of the Ca^{2+} and planar cell polarity (PCP) pathways. Canonical WNT signaling focuses on the role of β-catenin – a structural protein that forms a major component of adherens junctions and is also a critical transcriptional coactivator required for activation of WNT target genes. The astounding complexity described above with respect to the diversity of WNT ligands, receptors, coreceptors, and modulators could account for much of the diversity of action of WNT signaling. However at least canonical WNT signaling converges on β-catenin to affect transcriptional outputs and influence cell fate

decisions. It has been suggested that certain WNT proteins act canonically while others act non-canonically. It might be better to abandon this idea as even so called non-canonical WNTs such as wnt5a act canonically in different contexts (L. Gross, 2006; He et al., 1997; Mikels & Nusse, 2006; K. Willert & Nusse, 2012). It has been suggested that a more comprehensive view of WNT signaling is required obviating the distinction between the two categories of signaling and embracing a unified view of WNT signaling as 'cell fate' and 'cell polarity' divisions (Loh, Van Amerongen, & Nusse, 2016). The focus of this work is on the activity and effects of β -catenin, non-canonical WNT signaling effects will not be further discussed.

The molecular events of canonical WNT signaling focus on the fate of β -catenin. In the absence of the presence of a WNT ligand, B-catenin is continually signaled for destruction by a group of proteins aptly named the destruction complex. The destruction complex is composed of several proteins including Axin, adenomatous polyposis coli (APC), the serine/threonine kinases GSK3, casein kinase 1 (CK1), protein phosphatase 2A (PP2A), and the beta-transducin repeat containing E3 ubiquitin-protein ligase (BtrCP) (J. L. Stamos & Weis, 2013). Axin is a scaffold protein that contains binding sites for CK1, GSK3 and β-catenin, serving to bring the serine/threonine kinases CK1 and GSK3 in closer proximity to β-catenin (Yamamoto et al., 1999). Axin aids in the sequential phosphorylation of β -catenin by CK1 then GSK3 on multiple residues. Phosphorylated β-catenin is then ubiquitinated by BtrCP where it is signaled for degradation by the proteasome. APC is a critical component of the destruction complex, mutations in this protein rendering it nonfunctional lead to aberrant β-catenin accumulation and the subsequently leads to initiation of colorectal cancer, though the exact molecular mechanism remains incompletely understood (Roel Nusse & Clevers, 2017).

In response to WNT ligand binding the destruction complex is sequestered rendering it unable to phosphorylate β-catenin for degradation, this occurs through the following steps. In canonical WNT signaling, the protein DVL aids in the polymerization of FZD and LRP in response to binding of a WNT ligand and aids in the recruitment of Axin to the membrane (Bilic et al., 2007; Gao & Chen, 2010; Zeng et al., 2008). Since Axin is the main structural component of the desctruction complex, this effectively renders it ineffective at the cell membrane. Following

recruitment of Axin to the membrane β-catenin is hypophosphorlyated enabling its escape from degradation, it then accumulates in the cell cytoplasm, and translocates to the nucleus to affect transcription (Bilic et al., 2007; Gao & Chen, 2010; Zeng et al., 2008).

1.2.5. TCF/LEF transcription factors

β-catenin interaction with TCF/LEF transcription factors was initially identified from yeast two-hybrid screens (Behrens et al., 1996). TCF1 (HUGO gene name TCF7) and LEF1 transcription factors were discovered through efforts to identify transcriptional regulators of cell fate in human T lymphocytes (Travis, Amsterdam, Belanger, & Grosschedl, 1991; M van de Wetering, Oosterwegel, Dooijes, & Clevers, 1991). The final two TCF transcription factors in human, TCF3 (HUGO gene name TCF7l1) and TCF4 (HUGO gene name TCF7l2) were identified using low stringency hybridization screens with TCF7 cDNA (Vladimir Korinek et al., 1998). Although these factors were initially characterized as part of the immunology field, subsequent knockout studies have demonstrated their global roles in development and disease (Archbold, Yang, Chen, & Cadigan, 2012; Cadigan & Waterman, 2012a). Almost all invertebrates have at least one copy of a TCF/LEF transcription factor, while humans have four distinct TCF/LEF TFs, and zebrafish have five (Archbold et al., 2012). Additional complexity in WNT signaling is conferred by the additional TCF/LEF transcription factors (TFs) allowing different reactions in response to WNT signaling depending on the availability of different TCF/LEF TFs. The structure of TCF/LEF factors is well characterized, with an n-terminal β-catenin binding domain, HMG domain, basic tail, and c-clamp regions. Mutations in n-terminus result in dominant negative phenotypes similar to wg knockout mutants, additional evidence which demonstrates their interaction with β-catenin (Marc van de Wetering et al., 1997). Together the HMG domain and basic tail confer the ability to bind the well-characterized 5'-SCTTTGATS-3' consensus motif (Atcha et al., 2007; Hallikas & Taipale, 2006; van Beest et al., 2000; Marc van de Wetering et al., 1997). The HMG domain recognizes to and binds this consensus motif in the minor groove of the DNA and elicits a 90-127 degree bend in the DNA (Giese, Amsterdam, & Grosschedl, 1991; Love et al., 1995). The basic tail, a short sequence of amino acids serves as a nuclear localization

signal and enhances DNA binding (Giese et al., 1991; Prieve, Guttridge, Munguia, & Waterman, 1998).

The standard model of WNT mediates transcription suggests that in the absence of β-catenin binding TCF/LEF transcription factors act as inhibitors while binding of β-catenin induces transcription of WREs, this model is supported by studies in invertebrates such as drosophila/worms with a single TCF/LEF transcription factor (PAN – drosophila, POP1 nematode) (Brunner, Peter, Schweizer, & Basler, 1997; Cavallo et al., 1998; J. Liu, Phillips, Amaya, Kimble, & Xu, 2008; Marc van de Wetering et al., 1997). Additional specialized TCF/LEFs present in vertebrates which allow multiple interpretations of WNT signal, generating additional complexity. Generally TCF1 and LEF1 are linked to gene activation (F. Liu, van den Broek, Destrée, & Hoppler, 2005; T Reya et al., 2000) while TCF3 and TCF4 are linked to gene repression (C. H. Kim et al., 2000; Merrill et al., 2004). Although all four have been demonstrated to have activating potential, LEF1 and TCF3 seem to more reliably act as activator and repressor respectively, while TCF1 and TCF4 seem to alternate their function based on cell and tissue specific contexts (Galceran, Fariñas, Depew, Clevers, & Grosschedl, 1999; V Korinek et al., 1998; Nguyen et al., 2009; Roose et al., 1999; Tang et al., 2008). Interestingly a mechanism has been observed where TCF3 is phosphorylated and disassociates from DNA, which allows TCF1 to bind and activate gene expression (Hikasa et al., 2010; Hikasa & Sokol, 2011). Multiple TCF/LEFs have evolved specialized, complex, and context specific mechanisms to control the effects of canonical WNT signaling.

In the absence of β-catenin binding, TCFs have a repressive effect on WREs as discussed above. This effect is mediated by Groucho/TLE binds which acts as a repressive cofactor by binding TCF/LEF TFs to repress WNT target gene expression (Brantjes, Roose, van De Wetering, & Clevers, 2001; Cinnamon & Paroush, 2008; Turki-Judeh & Courey, 2012). TLEs form homotetramers, which both associate with nucelosomes and recruitment of HDACs to exert their repressive effects (Chodaparambil et al., 2014). HDAC recruitment by TLEs removes acetylation resulting in chromatin compaction and transcriptional repression (Ramakrishnan, Sinha, Fan, & Cadigan, 2018). β-catenin is hypothesized to out compete and displace TLE to recruit chromatin

activators and activate gene expression (Chodaparambil et al., 2014). Indeed, there appears to be no competition between B-catenin and TLE, as ChIP experiments shows that B-catenin and TLE1 are mutually exclusive in their binding patterns, suggesting there must be some sort of mechanism that facilitates exchange of the two (Sierra, Yoshida, Joazeiro, & Jones, 2006). Future work elucidating this mechanism will provide great insight into the regulation of WNT target genes.

TCF/LEFs are the dominant nuclear receptors for β-catenin, indeed some studies suggest that β -catenin binds exclusively to these factors (Schuijers, Mokry, Hatzis, Cuppen, & Clevers, 2014). Although evidence from several other groups indicates β-catenin may bind through alternate transcription factors including FOXO, SOX, PROP1, PITX2, HIF1a, and MYOD. Recent work provides strong evidence of TCF/LEF independent binding through what is termed a GHOST response (Doumpas et al., 2019). In this study all four TCF/LEF transcription factors were removed using CRISPR-cas9, then β-catenin ChIP-seq was performed. Results indicate β-catenin binding and activation of some subset of genes in the absence of these classically viewed fundamental transcription factors. The authors suggest different classes of β-catenin peaks, those entirely independent of TCF/LEF binding and those that use TCF/LEF as cofactors with other transcription factors. These studies demonstrate that the role of β-catenin as a transcriptional scaffold and activator continues to evolve.

1.2.6. β-catenin Structure and Function

β-catenin is the focus of canonical WNT signaling and of this project as a whole. It was originally identified as a component of adherens junctions, one of three proteins given the latin name for chain, catein (Ozawa, Baribault, & Kemler, 1989). The signaling function of β-catenin (armadillo) was discovered independently in a screen of mutations in drosophila searching for segment polarity genes (Nüsslein-Volhard, Wieschaus, & Kluding, 1984). Later analysis indicated that β-catenin (armadillo) protein accumulated in identical patterns to wg, indicating its role in linking the signaling pathway to segment polarity. Now it is well known that β-catenin serves dual roles, one as a major component of adherens junctions and second as the main transcriptional activator of Wnt target genes (Mosimann, Hausmann, & Basler, 2009b; Orsulic & Peifer, 1996;

Tomas Valenta, Hausmann, & Basler, 2012). The structure of β-catenin is what allows it to serve these two disparate roles in cells. The central region of β-catenin is made of 12 armadillo repeats that are flanked by an n terminal domain (NTD) and C- terminal domain (CTD) (Tomas Valenta et al., 2012). The central armadillo region forms a long positively charged groove that facilitates binding to E-caderin at cell junctions, AXIN/APC in the cytoplasm, and TCF/LEF in the nucleus (Huber, Nelson, & Weis, 1997). Multiple studies have begun to parse apart the different functions of β-catenin in a cellular context. Lyashenko et. al. have demonstrated that β-catenin was dispensable for maintenance of mESC pluripotency (Lyashenko et al., 2011). Reintroduction of a signaling deficient β-catenin rescued the ability to differentiate into ectoderm and endoderm but not mesoderm, indicating the differential requirements of β-catenin function in different germ layers. In a similar strain Valenta et al created signaling deficient β-catenin by mutating the N- and C- termini to examine the differential role of β-catenin in the developing dorsal neural tube, where they demonstrated the essential activity of transcriptional β-catenin to maintain progenitor identity and neuronal differentiation in the dorsal spinal cord (Tomas Valenta et al., 2011). β-catenin can be thought of as a scaffolding protein due to the many different proteins that it can bind to and recruit in different cellular contexts once bound to TCF/LEF in the nucleus. At the N-terminus it recruits BCL9 which in turn recruits pygopus, known to be required for transcriptional activity in drosophila WNT signaling. The C-terminus recruits many different transcriptional activators including the PAF1 complex, mediator complex, RNAPII PIC complex, Brg1, ISW1, HATs (TRRAP p400 and TIP60), and the SET1 COMPASS complex (Tomas Valenta et al., 2012). Competing mechanisms are hypothesized to explain the binding dynamics of the abundance of cofactors that are demonstrated to bind to the CTD of β-catenin. First there is the ping pong hypothesis, which states that the R11-C and Pygo NHD could provide a ping pong like surface to facilitate exchange of cofactors on the C-terminus. Alternatively there could be pools of β-catenin bound to various complexes before binding TCF/LEF, these complexes could then recruited sequentially. These hypotheses tend to suggest that β-catenin acts broadly to affect the transcriptional state of chromatin while interacting with tissue specific transcription factors to elicit transcription of target genes (Mosimann, Hausmann, & Basler, 2009a).

1.2.7. β-catenin as a transcriptional activator

Multiple studies have used ChIP-seq to examine the genome wide binding of β-catenin to understand its function in disease and development. Aberrant accumulation of β-catenin and activation of WNT signaling is the primary cause of colorectal cancer pathogenesis; to that end multiple studies have characterized β-catenin binding in cell culture systems of colorectal cancer (Bottomly, Kyler, McWeeney, & Yochum, 2010; Fodde, 2002; Schuijers et al., 2014; Watanabe et al., 2014). These studies establish a picture of β-catenin regulated genes binding through TCF/LEF transcription factors to influence colon carcinogenesis (Schuijers et al., 2014; Watanabe et al., 2014). β-catenin ChIP-seq studies in developing xenopus indicate roles for β-catenin as a cofactor for binding in gastrointestinal organogenesis and the developing gastrula (Nakamura, de Paiva Alves, Veenstra, & Hoppler, 2016; Stevens et al., 2017). Several studies use a human pluripotent stem cell derived cell culture system of early development to study β-catenin's transcriptional role in primitive streak development and mesendodermal differentiation (Estarás et al., 2015; Funa et al., 2015). Perhaps the most comprehensive view of β-catenin mediated transcriptional process is demonstrated by Estaras et al. where they demonstrate that β-catenin binds to and regulates the enhancer landscape forming enhancer promoter loops to regulate mesendeodermal gene expression (Estarás et al., 2015). β-catenin plays a role in multiple aspects of development a role which seems to be conferred by its ability to interact with context specific cofactors.

1.2.8. WNT signaling in Development and Disease

As evidenced by the many different developmental contexts which β-catenin demonstrated in ChIP-seq studies above, WNT signaling play an abundance of critical roles during development. This is perhaps best illustrated by the severe phenotypes observed when almost any component of WNT signaling is mutated. WNT mutant KOs have been extensively characterized in mouse systems. KO of WNT1 results in neural crest deficiencies and aberrant midbrain patterning (Ikeya, Lee, Johnson, McMahon, & Takada, 1997). Conditional mutation of β-catenin driven by Wnt1 affects cell fate in the dorsal spinal cord (T. Valenta et al., 2011). While KO of WNT1 in conjunction with Wnt4 results in a decrease in thymocyte production (Mulroy,

McMahon, Burakoff, McMahon, & Sen, 2002). Wnt3 plays roles in gastrulation and development of limbs (Barrow et al., 2003; C. Liu et al., 1999). Wnt3a KO generates mutants similar to Wnt1 with deficiencies in neural crest derivatives and CNS progenitors (Ikeya et al., 1997). Additionally Wnt3a plays roles in development of the hippocampus and in somitogenesis (Aulehla et al., 2003; Lee, Tole, Grove, & McMahon, 2000). Wnt4 is required for female germ line development, with its loss the mullerian duct is absent and there are defects in adrenal gland development (Vainio, Heikkilä, Kispert, Chin, & McMahon, 1999). Wnt5a plays multiple roles in development, including limb formation, lung morphogenesis, intestinal elongation, and mammary gland development (C. Li, Xiao, Hormi, Borok, & Minoo, 2002; Liang et al., 2003; Yamaguchi, Bradley, McMahon, & Jones, 1999; J. Yang, Wu, Tan, & Klein, 2003). Wnt7a deficiency results in infertile females due to abnormal development of mullerian duct derivatives (Timmreck, Pan, Reindollar, & Gray, 2003). Additionally evidence suggests Wnt7a acts as a synaptogenic factor, as its loss delays maturation of synapses in the cerebellum (Hall, Lucas, & Salinas, 2000). Wnt11 is known to regulate uteric branching in kidney development by directing the development of nephron progenitors (Majumdar, Vainio, Kispert, McMahon, & McMahon, 2003; O'Brien et al., 2018). The role of many WNTs have been investigated and characterized during development demonstrating its near ubiquitous appearance in an abundance of developmental contexts.

Due to the diverse set of processes in development that WNT signaling regulates, when processes in the pathway are misregulated they commonly result in a variety of diseases. Many WNT signaling defects result in different types of cancers (Zhan, Rindtorff, & Boutros, 2017). The classic example of WNT signaling is in familial adenomatous polyposis (FAP); mutations in APC were found to be the cause of this hereditary colon cancer (Kinzler et al., 1991; Nishisho et al., 1991; Rubinfeld et al., 1993). WNT signaling plays a well-known role in self-renewal and differentiation of hematopoietic stem cells (HSCs) (T C Luis, Ichii, Brugman, Kincade, & Staal, 2012; Tannishtha Reya et al., 2003). Most leukemia is characterized by abnormally high WNT signaling, with translocation products in AML frequently positively affecting WNT signaling (Cheng et al., 2008; Lane et al., 2011; Lento, Congdon, Voermans, Kritzik, & Reya, 2013; Muller-Tidow et al., 2004). WNT signaling additionally plays important roles in skin development (Fuchs, 2007).

Hyperactive β-catenin is implicated in melanoma tumor initiation with its decrease unexpectedly important in more malignant and metastatic tumors (Lim & Nusse, 2013). WNT signaling is also an important factor in breast cancer, with β-catenin mediated Cyclin1 D1 expression implicated in formation and progression (Lin et al., 2000). WNT signaling plays roles in many types of cancers' initiation and progression and modulating WNT signaling activity continues to be a main therapeutic goal for the cancer field.

In addition to the many types of cancers caused by misregulated Wnt signaling, there are a variety of developmental genetic disorders caused by defects in WNT signaling. Perhaps the most striking is the loss of Wnt3a, which results in tetra-amelia syndrome – loss of all four limbs (Niemann et al., 2004). Mutations in Wnt1 are linked to osteogenesis imperfecta, a disease characterized by reduced bone mass and increased susceptibility to recurrent fractures. (Fahiminiya et al., 2013; Keupp et al., 2013; Pyott et al., 2013) SERKAL (SEx Reversion, Kidneys, Adrenal, and Lung dysgenesis) syndrome were found to be caused by mutations in Wnt4, symptoms are consistent with mouse KO studies including female to male sex reversal and disrupted organogenesis. Similarly several disorders characterized by abnormal limb development such as Fuhrmann syndrome and Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome are characterized by mutations in Wnt7, and mouse KO studies demonstrate similar phenotypes (Parr & McMahon, 1995; Woods et al., 2006). WNTs play critical roles in development and adult homeostasis. Abnormalities in the pathway can result in severe developmental disorders and mutations that promote constitutive activation lead to cancers. Improved understanding of this complex signaling cascade could improve efforts to develop targeted therapeutics to combat the many diseases affected by this pathway.

1.3. Neural Development

"What is perhaps the most intriguing question of all is whether the brain is powerful enough to solve the problem of its own creation" (Eichele, 1992). The central nervous system is perhaps the most complex of all biological systems with an estimated more than 100 million neurons and glial cells forming more than 60 trillion neuronal connections (Stiles & Jernigan,

2010; Williams & Herrup, 1988). Here follows a brief outline of the current state of knowledge of neural development with an emphasis on the role of WNT signaling in these processes.

The central nervous system develops from the dorsal epiblast of the vertebrate gastrula (Wurst & Bally-Cuif, 2001). Early transplantation of the upper dorsal blastopore lip in amphibians by Spemann and Mangold led to the induced formation of a secondary embryo, including a neural plate of host cells, implicating the transplant as an organizing factor in neural induction (Spemann & Mangold, 2001). Analogous structures have since been identified in chick, fish, rabbit, and mouse suggesting a conserved mechanism of induction (Beddington, 1994; Waddington, 1950). However the molecular nature of the Spemann-Mangold organizer proved elusive for many years after its initial discovery. Eventually it was discovered that the Spemann organizer provided BMP antagonism by releasing BMP inhibitors Noggin (Lamb et al., 1993; Smith & Harland, 1992), chordin (Sasai, Lu, Steinbeisser, & De Robertis, 1995), and follistatin (Hemmati-Brivanlou, Kelly, & Melton, 1994). This led to the idea of a model of default neural induction in the ectoderm, that in the absence of BMP signaling neural ectoderm was induced while BMP induced epidermis (Muñoz-Sanjuán & Brivanlou, 2002). This idea has since been challenged by studies that demonstrate role for FGF in neural induction independent of BMP in both xenopus (Delaune, Lemaire, & Kodjabachian, 2005) and chick (Linker & Stern, 2004). The role for WNT signaling is still under dispute perhaps due to variable signaling requirements among species (D. Brafman & Willert, 2017; Stern, 2005).

1.3.1. Neural induction

Several studies have demonstrated in xenopus that WNT signaling is required for neural induction (Baker, Beddington, & Harland, 1999; Wessely, Agius, Oelgeschläger, Pera, & De Robertis, 2001). In these instances WNT signaling was shown to repress BMP signaling, either directly (Baker et al., 1999) or by inducing expression of BMP antagonists (Wessely et al., 2001), to cause neural induction. Multiple studies however contradict this earlier work, demonstrating that WNT signaling interferes with neural induction and active WNT signaling is not required (Glinka et al., 1998; Heeg-Truesdell & LaBonne, 2006; Min, Kriebel, Hou, & Pera, 2011; S. I.

Wilson et al., 2001). Future work could perhaps reconcile these contradictory results, however it is well agreed that BMP antagonism results in the neutralization of naïve ectoderm.

The sequential two step activation, transformation model of neural induction and subsequent patterning was put forth by Nieuwkoop et al. following experiments where competent ectoderm was transplanted into different antero-posterior levels of the neural plate (Nieuwkoop, 1952). This hypothesis has gained considerable support, with the Spemann organizer leading to the activation, or induction of neural ectoderm with an anterior fate and a gradient of WNT signaling found to specify the regional cell identities along the antero-posterior axis (Kiecker & Niehrs, 2001). Ectopic expression of xWnt3a in xenopus animal caps reduced expression of anterior neural genes and elevated expression of posterior neural genes, providing the first evidence that WNT serves as a transforming posteriorizing factor in specification of regional identity of neural progenitors (McGrew, Lai, & Moon, 1995), Loss of function studies using a dominant negative form of Xwnt8 supported these results.(Bang, Papalopulu, Goulding, & Kintner, 1999; McGrew, Hoppler, & Moon, 1997). Although several different organizer structures have been hypothesized over the years to coordinate induction and patterning of the neural tube, the source of the WNT gradient that regulates A/P fate has been identified to come from the paraxial dorsolateral mesoderm, beneath the developing neural tube (Elkouby et al., 2010). The headless (hdl) zebrafish mutant, as its moniker suggests, is missing the anterior neural structures including the eyes, forebrain, and part of the neural tube. This phenotype is a result of a mutation in TCF3, a negative regulator of WNT signaling, further demonstrating the importance proper regulation of WNT signaling in generating a properly patterned neural tube (C. H. Kim et al., 2000). Additional studies have estabished the importance of WNT inhibition in generation of anterior neural structures including Dickkopf-1 (Dkk1) (Glinka et al., 1998; Hashimoto et al., 2000; Kazanskaya, Glinka, & Niehrs, 2000; Kimura-Yoshida et al., 2005; Mukhopadhyay et al., 2001) and secreted frizzled receptor proteins (SFRPs) (Kemp, Willems, Abdo, Lambiv, & Leyns, 2005; Leyns, Bouwmeester, Kim, Piccolo, & De Robertis, 1997; Mii & Taira, 2009).

1.3.2. WNT and patterning of the forebrain

As previously discussed, WNT inhibition is required for forebrain development and this requirement continues for patterning of the telencephalon (giving rise to the cerebral cortex), while the more posterior diencephalon (giving rise to the thalamic tissues) requires local WNT signaling to elicit the appropriate cell fate (S. W. Wilson & Houart, 2004). Several studies have demonstrated the need for repression of WNT signaling for proper telencephalon development, when WNT signaling is high due to ectopic expression or genetic mutations, telencephalic identity is repressed and an expanded diencephalon is observed (R. Quinlan, Graf, Mason, Lumsden, & Kiecker, 2009). WNT signaling also is known to affect dorsal (pallidal) vs ventral (subpallidal) cell fates in the developing forebrain, with higher levels of WNT signaling corresponding to correct dorsal patterning (Campbell, 2003; Gulacsi & Anderson, 2008; Gunhaga et al., 2003). The dorsal telencephalon develops into the cerebrum, part of which is composed of the hippocampus (Hébert & Fishell, 2008). WNT signaling is a demonstrated requirement for hippocampus development (Galceran, Miyashita-Lin, Devaney, Rubenstein, & Grosschedl, 2000; Lee et al., 2000). A proper balance of repression and activation of WNT signaling is necessary for forebrain patterning.

1.3.3. WNT and patterning of the midbrain

Specification of midbrain structures is strongly dependent on WNT1 expression, as mouse knockout studies of this gene have demonstrated dramatic loss of midbrain and rostral hindbrain structures (A P McMahon, Joyner, Bradley, & McMahon, 1992; Andrew P. McMahon & Bradley, 1990; K R Thomas & Capecchi, 1990; Kirk R. Thomas, Musci, Neumann, & Capecchi, 1991). Later studies have shown that the isthmic organizer or midbrain hindbrain boundary is a key organizer regulating formation of the midbrain and anterior hindbrain, a balance dependent both WNT and FGF signaling (C. Guo et al., 2007; H. Harada, Sato, & Nakamura, 2016; Rhinn & Brand, 2001). Conditional knockout of b-catenin under the Wnt1 regulatory sequence provided supporting evidence of the role of Wnt1 in the formation of the midbrain and cerebellum and an unexpected function in craniofacial development suggesting a role in neural crest development (Brault et al., 2001). Future work further examined the role of β-catenin in midbrain development

by creating β-catenin mutations in the N- and C- termini to examine the effects of β-catenin in adhesion vs. transcriptional activation in midbrain development (Tomas Valenta et al., 2011). Disruption of the signaling ability of β-catenin resulted in reduced expression of Pax3 and Sox2 in the dorsal neural tube, demonstrating the importance of β-catenin transcription in mediating cell fate decisions in the dorsal neural tube (Tomas Valenta et al., 2011).

1.3.4. WNT and patterning of the hindbrain

WNT also plays important roles in the generation of the hindbrain and spinal cord. Wnt3a has been shown to activate meis3, a critical transcription factor required for hindbrain induction (Elkouby et al., 2010; Elkouby, Polevoy, Gutkovich, Michaelov, & Frank, 2012). In the absence of WNT signaling Meis3 is able to induce hindbrain generation (Elkouby et al., 2010). Although WNT signaling is essential for generation of initial patterning of hindbrain structures; future subdivisons are refined based on FGF and RA signaling (Esain, Postlethwait, Charnay, & Ghislain, 2010; Ishioka et al., 2011; Mazzoni et al., 2013; Partanen, 2007). Similarly to its action in the hindbrain, WNT signaling also regulates induction and patterning of the spinal cord (D. Brafman & Willert, 2017). WNT action in spinal cord generation is thought to act through CDX transcription factors, which serve as the central mediators of the gene regulatory network in the spinal cord (Lohnes, 2003). CDX genes are direct WNT targets with TCF/LEF binding sites in their gene regulatory regions (Haremaki, Tanaka, Hongo, Yuge, & Okamoto, 2003; Lickert & Kemler, 2002; Pilon, Oh, Sylvestre, Savory, & Lohnes, 2007; Prinos et al., 2001; W. C. H. Wang & Shashikant, 2007). In fact, similar to Meis3 activity in the hindbrain CDX2 can substitute for WNT to induce caudalization of neural cells to a spinal cord fate (Metzis et al., 2018).

WNT signaling plays roles in induction, initial A/P patterning, and further refinement of subdivisons of the developing neural tube. Therefore it is not surprising that aberrant WNT signaling can lead developmental disorders. Both canonical and non-canonical WNT signaling are implicated in neural tube defects (NTD) that can lead to conditions such as spina bifida, anencephaly, and craniorachischisis (Mulligan & Cheyette, 2012). Additionally mutations in the WNT signaling pathway have been implicated in a variety of psychiatric disorders, including autism spectrum disorder (ASD) (Gilman et al., 2011; Krumm, O'Roak, Shendure, & Eichler,

2014; P.-M. Martin et al., 2013; Mohn et al., 2014; O'Roak et al., 2012; Turner et al., 2016; Wassink et al., 2001), schizophrenia (Hoseth et al., 2018; Panaccione et al., 2013), and bipolar disorder (BD) (Muneer, 2017; Valvezan & Klein, 2012). Emerging cerebral organoid technologies offer the opportunity to study the effects of these various mutations during neural development to understand how mutations in the WNT signaling pathway may be implicated in the organization of brain structures leading to psychiatric disorders (Hansen Wang, 2018; Wen, Christian, Song, & Ming, 2016).

1.4. Chromatin and Epigenetics

The first use of the term epigenetics can be attributed to C.H. Waddington, where he defined it as the 'causal mechanism by which the genes of the genotype bring about phenotypic effect' (Waddington, 2012). The definition of epigenetics has since been expanded and refined, which has led to diverging definitions at times (Deans & Maggert, 2015; David Haig, 2012). The differences in definitions ascribed to epigenetics could be due to the dual origins of the word (D Haig, 2004). In the original sense the term described two concepts that Waddington pioneered, phenotypic plasticity and canalization (Deans & Maggert, 2015). Phenotypic plasticity refers to the ability of individual genotypes to produce different phenotypes (Pigliucci, Murren, & Schlichting, 2006). Canalization describes the property of stability of phenotype across different genotypes and environments. (Waddington, 1959) Some years later David Nanney described epigenetics as a cellular control system separate from the genotype that consisted of supporting mechanisms to control the expression of specific genes that persist after cell division (Deans & Maggert, 2015; Nanney, 1958). Following studies examining cellular memory and DNA methylation, Holliday proposed a new definition of epigenetics as nuclear inheritance, not based on differences in DNA sequence (Holliday, 1994). This definition was refined by Wu and Morris as the study of changes in gene function that are mitotically or meiotically heritable and that do not entail change in DNA sequence (Wu Ct & Morris, 2001). It is this definition that the following work will focus on.

1.4.1. The structure and organization of chromatin

Chromatin is the complex of DNA and protein located in the nucleus that compacts DNA to regulate which genes are transcribed into RNA is a cell type specific way (Perino & Veenstra,

2016). The proteins that complex with DNA to form chromatin are histones, these were originally thought to act generally as inhibitors of transcription. (Huang & Bonner, 1962; Stedman & Stedman, 1950) X-ray and biochemical analysis revealed histones to form an octamer of two repeating units composed of the main four histones, H2A, H2B, H3, and H4 around which was wrapped about 200 bp of DNA. This repeating structure is known as a nucleosome (Kornberg, 1974, 1977; Kornberg & Thomas, 1974). X-ray crystallography studies provided detailed analysis of the structure of nucelosomes revealing that each histone consists of a three-helix domain known as the histone fold as well as two unstructured tails (K Luger, Mäder, Richmond, Sargent, & Richmond, 1997; Rhodes, 1997; Richmond, Finch, Rushton, Rhodes, & Klug, 1984). Each core octamer is connected by the linker histone H1 that binds 20 to 75 bp of DNA, which is thought to stabilize higher-order chromatin structures (Allan, Hartman, Crane-Robinson, & Aviles, 1980; Oudet, Gross-Bellard, & Chambon, 1975; Woodcock, Skoultchi, & Fan, 2006). The repeating 'beads on a string' structures formed by nucelosomes impedes transcription of DNA by physical obstruction and bending the DNA to reduce availability to transcription (K Luger et al., 1997).

Chromatin has long been described has having hierarchical structures with progressively higher order and more densely compacted structures (Grigoryev, 2018; Ou et al., 2017). As described above the smallest unit is the 11 nm DNA-core nucleosome structure, these assemble into 30 nm fibers, then 120 nm chromonema, 300-700 nm chromatids, and finally the most condensed structural form occurs the in metaphase chromosomes (Ou et al., 2017). Recent studies however question this hierarchical structure hypothesis, as several imaging technologies have been unable to visualize this different sized fibers (Eltsov, Maclellan, Maeshima, Frangakis, & Dubochet, 2008; Fussner et al., 2012; Nishino et al., 2012). The development of a novel imaging technology called ChromEMT has allowed visualization of fixed chromatin in the cell nucleus, in comparison to previous studies which were based on chromatin that had been reassembled in vitro by reconstituting purified DNA and histones (Ou et al., 2017). This technology has revealed that chromatin in cells doesn't tend to form a hierarchical structure and instead adopts a disordered 5 to 24 nm diameter chain packed together at different densities throughout the nucleus (Ou et al., 2017). Future work visualizing intact chromatin could enable

the direct mapping of proteins in chromatin providing great insight into the way chromatin structure is regulated and suggesting mechanisms for manipulation (Larson & Misteli, 2017).

1.4.2. Chromatin structure as a mechanism of transcriptional control

Chromatin is generally thought of as in an open or closed state relative to transcription, these are known as euchromatin or heterochromatin respectively (Perino & Veenstra, 2016). Euchromatin and heterochromatin states are regulated by the density of nucelosomes, histone variant incorporation, and post transcriptional modifications (PTM) of histone tails and body (Karolin Luger, 2006). The density of nucleosomes can be controlled by ATP dependent chromatin remodeling complexes (Clapier, Iwasa, Cairns, & Peterson, 2017). Additionally chromatin state can be controlled by histone variants incorporated into the core nucleosome structure (Karolin Luger, 2006; Widom, 1998). For example the histone variant H2A.Z is more amenable to compaction than the canonical H2A variant (Fan, Gordon, Luger, Hansen, & Tremethick, 2002). Finally there are PTMs of histone N-terminal tails which can affect histone-DNA or histone-histone contacts to influence chromatin accessibility (Bowman & Poirier, 2015). Acetylation of H3 on lysine residues is perhaps the most well studied example of this phenomenon, a mark that is associated with increased transcription at promoters due to the charge of the acetyl group repelling DNA from the nucleosome core (Brownell et al., 1996; Grunstein, 1997; Taunton, Hassig, & Schreiber, 1996). The role of common PTMs has been extensively characterized, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP-ribosylation (Bannister & Kouzarides, 2011; Rothbart & Strahl, 2014; Wozniak & Strahl, 2014). The diversity of PTMs that can be deposited on both histone tails and globular domains give rise to a complex histone code which influences chromatin structure and thus transcriptional availability of DNA (Janssen, Sidoli, & Garcia, 2017).

PTMs are dynamic reversible modifications that are deposited by enzymes known as histone writers and removed by histone erasers (Gillette & Hill, 2015). Readers and writers are separated into classes based on the specific PTM that they regulate. Additionally there are histone readers which are proteins that recognize PTMs to effect specific transcriptional outcomes (Yun, Wu, Workman, & Li, 2011). Lysine acetylation is one of the most characterized

PTMs, it is deposited by histone acetyltransferases (HATs) and removed by histone deacetlyases (HDACs) (Yun et al., 2011). Acetylated lysines are recognized by bromodomains, which regulate initiation and elongation of transcription (Dhalluin et al., 1999; Josling, Selvarajah, Petter, & Duffy, 2012). Lysine methylation is another of the most common PTMs present in cells, there are four types of methylated marks: unmethylated, mono- (me1), di (me2), and tri- (me3) methylation (Yun et al., 2011). These marks are regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs) (Teperino, Schoonjans, & Auwerx, 2010). The four types of methylation that can be present on histones are recognized by many different types of protein domains including chromo, WD40, Tudor, double/tandem Tudor, MBT, Ankyrin Repeats, zf-CW, and PWWP domains (Yun et al., 2011). The general transcription factor TFIID, important in the assembly of the pre-initiation complex, additionally recognizes H3K4me3 (Vermeulen et al., 2010). Just as more PTMs continue to be discovered, information on writers, erasers, and readers of additional PTMs continues to be an area of active investigation.

hESCs have a globally open chromatin state with less heterochromatin and more active chromatin domains than differentiated cells (Azuara et al., 2006; Guenther, Levine, Boyer, Jaenisch, & Young, 2007; Lister et al., 2009; Meissner et al., 2008; Park et al., n.d.). Developmental genes in the ES state are also simultaneously marked with active (H3K4me3) and repressive (H3K27me3) marks at developmental genes, these genes described as bivalent and thought to be poised for expression (Azuara et al., 2006; Bernstein et al., 2006). As ES cells differentiate bivalent genes are resolved and become mono-valent, either active or repressed (Mikkelsen et al., 2007). Multiple studies have examined how chromatin profiles changes as cells adopt a more differentiated state, revealing the complex interplay of transcription factor binding, enhancer occupancy, and transcriptional state of developing cells (Dixon et al., 2015; Dowen et al., 2014; Heintzman et al., 2009; Ji et al., 2016; Tsankov et al., 2015; A. Wang et al., 2015; Xie et al., 2013). It's important to understand both genetic and epigenetic changes occurring during development, as most common variation in the human genome doesn't always affect protein coding genes, suggesting the effects of these differences could be attributed to changes in epigenetic state (Khurana et al., 2013).

The transcriptome of the developing and adult human brain has been characterized in some detail, outlining genetic circuits that underpin human neural development (Hawrylycz et al., 2012; Kang et al., 2011; Konopka et al., 2012; Miller et al., 2014; Silbereis, Pochareddy, Zhu, Li, & Sestan, 2016). Some enhancer networks have been identified in adult human brain, however information regarding the dynamic changes in chromatin structure during neural development have not yet been mapped (Vaidya & Chakradhar, 2014). Changes in histone occupancy at genetic elements such as enhancers are thought to contribute to cell competence, therefore affecting transcription factor occupancy and cell differentiation (Buecker & Wysocka, 2012; Metzis et al., 2018; A. Wang et al., 2015). Human ES and iPSC models of neural development will allow interrogation of epigenetic changes during neural development to enhance our understanding of how the epigenetic landscape is altered in healthy and diseased states during development (Lancaster et al., 2013).

1.5. Specific Aims

We propose to complete this study through the following specific aims:

1.5.1. Specific Aim1

Development and characterization of an in vitro model of neural patterning using human pluripotent stem cells

1.5.2. Specific Aim2

Characterization of the role of β-catenin in regulating fate decisions of human neural progenitor cells in neural patterning

1.5.3. Specific Aim3

Characterization of the role of negative regulators of WNT signaling regulating fate decisions of human neural progenitor cells in neural patterning

CHAPTER 2

DEVLOPMENT OF AN IN VITRO MODEL OF NEURAL DEVELOPMENT USING WNT SIGNALING

2.1. Introduction

Neural progenitor cells (NPCs) and neurons derived from human pluripotent stem cells (hPSCs) could provide an unlimited source of cells for drug testing and cell-based therapies (Koch, Kokaia, Lindvall, & Brüstle, 2009; S.-C. Zhang, Li, Johnson, & Pankratz, 2008). In addition, these cells provide a unique opportunity to explore complex neural development in a simplified and accessible system. Current protocols for differentiating hPSCs toward specific neuronal lineages result in a mixture of neurons from various regions of the CNS, which limits the use of these cells for cell-based therapies, disease modeling, and developmental studies that require uniform populations of neurons. However, the precise source of this heterogeneity in neuronal cultures has yet to be resolved.

Differentiation of stem and progenitor populations is largely governed by the heterogeneity present in these cultures, which ultimately determines their differentiation bias. For example, several studies have found subpopulations with distinct self-renewal and differentiation potentials in hematopoietic (Dykstra et al., 2007; S. Huang, Guo, May, & Enver, 2007) and intestinal (Sangiorgi & Capecchi, 2008) stem cells. Likewise, heterogeneous expression of pluripotency-related transcription factors and other cell-surface markers bestows distinct lineage-specific differentiation propensities on hPSCs (Drukker et al., 2012; Hong et al., 2011; Narsinh et al., 2011; Stewart et al., 2006; Wu & Tzanakakis, 2012). In contrast, NPCs derived from hPSCs have been considered to be a homogeneous cell population, and it has been suggested that their differentiation to neuronal cultures can be biased and manipulated by altering culture conditions (Dottori & Pera, 2008a; Gaspard & Vanderhaeghen, 2010a; Germain, Banda, & Grabel, 2010; Y. Jiang, Zhang, & Hu, 2012; H. Liu & Zhang, 2011; Nat & Dechant, 2011; Peljto & Wichterle, 2011; S. C. Zhang, 2006). Our study challenges this simplistic view of neuronal differentiation in hPSC cultures.

We demonstrate that hPSC-derived NPCs, like other stem and progenitor populations, are heterogeneous and display a bias in their differentiation potential. Through the use of WNT reporter hPSC lines, we identified endogenous WNT signaling as a primary regulator of this heterogeneity in NPC and neuronal cultures. Flow cytometry (FC)-based purification and genetic assessment of reporter-expressing cell types revealed that the identity and differentiation potential of hPSC-derived NPCs are directly related to the level of endogenous WNT signaling present in these cell types. Through exogenous manipulation of WNT signaling, we were able to reduce NPC heterogeneity and generate cultures of regionally specific progenitors and neurons. Overall, this study demonstrates that WNT signaling plays an important role in deriving regionally homogeneous populations of NPCs and neurons, thereby greatly improving their scientific and therapeutic utility.

2.2. Experimental Methods

2.2.1. Cells and culture conditions

All media components were from Life Technologies unless otherwise noted. For hPSC culture, the following media were used: mouse embryonic fibroblast (MEF) (1X high glucose DMEM, 10% fetal bovine serum, 1% (v/v) L-glutamine penicillin/streptomycin). H9/HES3/RiPSC hPSCs (1X DMEM-F12, 20% (v/v) Knockout Serum Replacement, 1% (v/v) non-essential amino acids, 0.5% (v/v) glutamine, 120 μM 2-mercaptoethanol [Sigma]). HUES9 (1 x Knockout DMEM, 10% (v/v) Knockout Serum Replacement, 10% (v/v) human plasmanate (Chapin Healthcare, Anaheim CA, USA) 1% (v/v) non-essential amino acids, 0.5% (v/v) glutamine, 55 μM 2-mercaptoethanol [Sigma]); All hPSC lines were maintained on feeder layers of mitotically inactivated MEFs (2x104 /cm2 ; Millipore). All hPSC cultures were supplemented with 30 ng/ml FGF2 (Life Technologies). MEF-CM was produced by culturing hPSC medium on MEFs for 24 hr followed by sterile filtering. Cells were routinely passaged with Accutase (Millipore), washed, and replated at a density 4.25 x 10^4 /cm^2 .

2.2.2. Generation of Wnt reporter hESCs

The lentiviral construct that was used to generate the WNT reporter line contained a 7xTCF-eGFP construct and puromycin resistance gene. (Fuerer & Nusse, 2010) High titer

lentivirus was produced as previously described (Miyoshi, Blömer, Takahashi, Gage, & Verma, 1998; Zufferey et al., 1998). HUES9 hESCs were infected overnight with lentivirus. Infected pools were selected with puromycin (0.5 µg/ml) for 2 weeks. For generation of clonal hESC lines, transduced pools were then treated with 200 ng/ml purified mouse WNT3a for 48 hours. Cells were dissociated with Accutase for 5 min at 37°C, triturated, and passed through a 40 µm cell strainer. Cells were then washed twice with FACS buffer (PBS, 10 mM EDTA, and 2% FBS) and resuspended at a maximum concentration of 5 x 106 cells per 100 ul. Single TOP-GFP+ cells were sorted into a Matrigel (BD)-coated 96 well plated with MEF-CM supplemented with 5 µM ROCK inhibitor Y27632 (Stemgent) and 30 ng/ml FGF2. After expansion, a total of 45 clones were screened for: (1) robust TOP-GFP expression upon WNT3a stimulation and (2) normal euploid karyotype.

2.2.3. Neural progenitor cell (NPC) generation, expansion, and differentiation

To initiate neural differentiation, hPSCs were cultured on Matrigel (BD Biosciences) in MEF-CM supplemented with 30 ng/ml FGF2 or TeSRTM2 (Stem Cell Technologies). Cells were then detached with treatment with Accutase (Millipore) for 5 min and resuspended in neural induction media (1% N2/1% B27 without vitamin A/DMEM:F12) supplemented with 5 µM Y-267632 (Stemgent), 50 ng/ml recombinant mouse Noggin (R&D Systems), 0.5 µM Dorsomorphin (Tocris Bioscience)]. Next, 7.5 x 105 cells were pipetted to each well of a 6-well ultra low attachment plates (Corning). The plates were then placed on an orbital shaker set at 95 rpm in a 37°C/5% CO2 tissue culture incubator. The next day, the cells formed spherical clusters (embryoid bodies [EBs]) and the media was changed to neural induction media with 50 ng/ml recombinant mouse Noggin and 0.5 µM Dorsomorphin. The media was subsequently changed every other day. After 5 days in suspension culture, the EBs were then transferred to a 10 cm dish coated (3 x 6 wells per 10 cm dish) with growth factor reduced Matrigel (1:25 in KnockOut DMEM; BD Biosciences) for attachment. The plated EBs were cultured in neural induction media with 50 ng/ml recombinant mouse Noggin and 0.5 µM Dorsomorphin for an additional 7 days. Neural rosettes were cut out by dissection under an EVOS (Life Technologies) microscope. Dissected rosettes were incubated in Accutase for 5 min and then triturated to single cells with a

1 mL pipet. Rosettes were then plated onto poly-Lornithine (PLO; 10 µg/mL; Sigma) and mouse laminin (Ln; 5 µg/mL; Sigma) coated dishes at a density of 12,500 cells/cm2 in neural induction media supplemented with 10 ng/mL mouse FGF2 and 10 ng/ml mouse EGF2 (R&D Systems). IWP2 (Stemgent) and CHIR 98014 (CHIR; Axon Medchem) were added 2 days after EB formation. For routine maintenance, NPCs were passaged onto PLO/Ln coated plates at a density of 10,000 cells/cm^2 in neural induction media supplemented with 10 ng/mL mouse FGF2 and 10 ng/ml mouse EGF2. TOP-GFP sorted as well as IWP2- and CHIR-treated NPCs were derived and maintained in the absence of FGF2 and EGF2. For neuronal differentiation, NPCs were dissociated with Accutase for 5 min at 37°C, triturated, and plated onto PLO/Ln coated plates at a density of 100,000 cells/cm^2. Cells were cultured in neuronal differentiation media (0.5% N2/0.5% B27 without vitamin A/DMEM:F12) supplemented with 20 ng/ml BDNF (R&D Systems), 20 ng/ml GDNF (R&D Systems), 1 µM DAPT (Tocris Bioscience), and 0.5 mM , dibutyrl-cAMP (db-cAMP; Sigma) for 4 weeks.

2.2.4. Quantitative PCR (qPCR)

RNA was isolated from cells using TRIzol (Life Technologies), and treated with DNase I (Life Technologies) to remove traces of genomic DNA. Reverse transcription was performed with qScript cDNA Supermix (Quanta Biosciences). Quantitative PCR was carried out using TaqMan probes (Life Technologies) and TaqMan Fast Universal PCR Master Mix (Life Technologies) on a 7900HT Real Time PCR machine (Life Technologies), with a 10 min gradient to 95°C followed by 40 cycles at 95°C for 15s and 60°C for 1 min. Taqman gene expression assay primers (Life Technologies; Table S4) were used. Gene expression was normalized to 18S rRNA levels. Delta Ct values were calculated as Ct target– Ct18s. All experiments were performed with three technical replicates. Relative fold changes in gene expression were calculated using the 2-ΔΔCt method. Data are presented as the average of the biological replicates ± standard error of the mean (S.E.M).

2.2.5. Immunofluorescence

Cultures were gently washed twice with staining buffer (PBS w/ 1% (w/v) BSA)

prior to fixation. Cultures were then fixed for 15 min at room temperature (RT) with fresh paraformaldeyde (4% (w/v)). The cultures were washed twice with staining buffer and permeabilized with 0.2% (v/v) Triton-X-100 in stain buffer for 20 min at 4°C. Cultures were then washed twice with staining buffer. Primary antibodies were incubated overnight at 4°C and then washed twice with stain buffer at RT. Secondary antibodies were incubated at RT for 1 hr. Antibodies used are listed in Table S5. Nucleic acids were stained for DNA with Hoechst 33342 (2 µg/ml; Life Technologies) for 5 min at room temperature. Imaging was performed using an automated confocal microscope (Olympus Fluoview 1000 with motorized stage). Quantification of images was performed by counting a minimum of 9 fields at 20x magnification. Image quantification of the data is presented as the average of these fields ± standard deviation (S.D.).

2.2.6. Flow cytometry and cell replating

Cells were dissociated with Accutase for 5 min at 37°C, triturated, and passed through a 40 µm cell strainer. Cells were then washed twice with FACS buffer (PBS, 10 mM EDTA, and 2% FBS) and resuspended at a maximum concentration of 5 x 106 cells per 100 ul. One test volume of antibody was added for each 100 µl cell suspension (Table S5). Cells were stained for 30 min on ice, washed, and resuspended in stain buffer. Cells were analyzed and sorted with a FACSCanto or FACSAria (BD Biosciences). Flow cytometry data was analyzed with FACSDiva software (BD Biosciences). Isotype negative controls are listed in Table S4. For sorting experiments in which cells were separated on the basis of GFP expression, wild-type (WT) non-fluorescing cells were used as a negative control. For replating experiments, cells were stained with appropriate antibodies and sorted into FACS buffer with 5µM Y27632 (Stemgent). Sorted cells were replated at the appropriate density and media with 10 µM Y27632

2.2.7. RNA sequencing (RNA-seq)

Total RNA from FACS sorted TOP-GFP+ and TOP-GFP- NPCs were isolated, depleted of genomic DNA and rRNA and fragmented to ~200 bp by RNase III. After ligating the Adaptor Mix, fragmented RNA was converted to the first strand cDNA by ArrayScript Reverse Transcriptase (Ambion), size selected (100-200bp) by gel electrophoresis, and amplified by PCR using adaptor-specific primers. Deep sequencing was performed on an Illumina Genome Analyzer II. Analysis of

genome-wide expression data was performed as previously described (Trapnell et al., 2013, 2012). Briefly, raw reads from two biologically independent samples were aligned to the reference human genome (hg19) using TopHat. Cufflinks was used to assemble individual transcripts from the mapped reads. Cuffmerge was used to merge the assembled transcripts from the two biologically independent samples. Cuffdiff was used to calculate gene expression levels and test for the statistical significance of differences in gene expression. Reads per kilobase per million mapped reads (RPKM) were calculated for each gene and used as an estimate of expression levels.

2.3. Results and Discussion

2.3.1. Endogenous WNT Signaling is a major source of heterogeneity in NPCs derived from hPSCs

It is well established that WNT signaling regulates the regional identity along the anterior-posterior (A/P) axis of the developing CNS. To explore the possibility that WNT signaling exerts similar effects in a cell-culture-based system of neural development, we generated clonal human embryonic stem cell (hESC) lines (HUES9) carrying a stably integrated GFP reporter under the control of a WNT-responsive promoter, called TCF Optimal Promoter (TOP) (Fuerer & Nusse, 2010) (Figure A.1A). In undifferentiated hESCs, this reporter is inactive but expresses GFP upon stimulation with recombinant WNT3a (Figure A.1B). In contrast to a previous study (Blauwkamp, Nigam, Ardehali, Weissman, & Nusse, 2012), none of our clones or the nonclonal pool expressed GFP in the absence of exogenous WNT3a. This likely reflects the heterogeneity among hESC lines, especially with respect to endogenous expression of WNT3 (W. Jiang, Zhang, Bursac, & Zhang, 2013). In a subsequent analysis we focused on one clone, clone 19 (hTOP-19), which exhibited robust GFP expression upon WNT3a stimulation (nearly 100%; Figure A.1B), displayed a normal female karyotype of 46 chromosomes (Figure A.1C), and responded to various concentrations of exogenously added WNT3a (Figure A.1D) and chemical inhibitors of GSK3β, such as BIO (Figure A.1E).

Upon differentiation of this WNT reporter line to NPCs (D. A. Brafman, 2015) (Figure A.2A), we observed a heterogeneous pattern of GFP expression in the absence of any exogenously

added WNT proteins (Figure 2.1A). Despite uniform expression of the pan-neural markers SOX1, SOX2, and NESTIN (Figures A.2B and A.2C), and cell morphology (Figure A.2D), FC revealed that GFP expression peaked upon rosette formation and a stable population of GFP-positive (GFP+) cells persisted through subsequent NPC passages (Figure 2.1B). Addition of WNT3a to these NPC cultures resulted in uniformly high GFP expression, thereby demonstrating a homogeneous response to WNT signaling in this clonally derived population (Figure A.1F). Furthermore, inhibiting endogenous WNT signaling with IWP2, a small molecule that acts on PORCN to block WNT processing and subsequent secretion (B. Chen et al., 2009), eliminated GFP expression, confirming that reporter expression was due to endogenous WNT signaling (Figure A.1F).

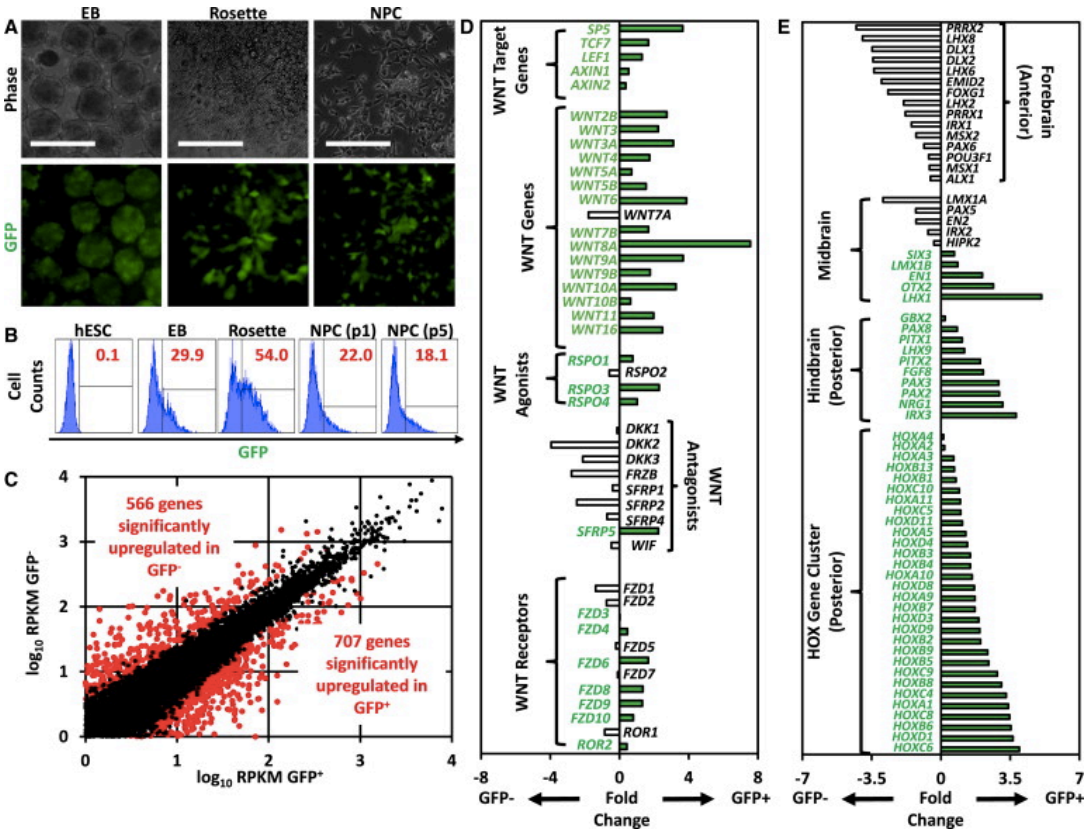

Figure 2.1 NPCs are heterogeneous with respect to endogenous WNT signaling A) Phase contrast and fluorescent images of WNT reporter hESCs during neural differentiation (EB, scale bar, 500 μm; rosette and NPC, scale bar, 200 μm). B) FC of WNT reporter hESCs during neural differentiation. No detectable GFP signal was observed in hESCs, but upon differentiation to

NPCs, a range of GFP expression patterns was observed. A population of GFP+ cells endured through multiple NPC passages. C) Passage 5 reporter-expressing NPCs were separated by FC on the basis of GFP expression. A scatterplot of log10 RPKM in GFP+ and GFP− NPCs is shown. Genes with statistically significant differences in expression are shown in red. D and E) Selection of differentially expressed genes highlighting differences in gene-expression patterns related to (D) WNT signaling and (E) A/P patterning and differentiation of the neural tube.

Consistent with this heterogeneous expression of WNT reporter activity and with previously published studies (Dottori & Pera, 2008b; Gaspard & Vanderhaeghen, 2010b; Germain et al., 2010; H. Liu & Zhang, 2011; Nat & Dechant, 2011; Peljto & Wichterle, 2011), we found that NPCs derived under this protocol exhibited significant heterogeneity with respect to regionally specific markers (Figures A.2E–A.2H) despite uniform expression of the pan-neural markers SOX1 and SOX2 (Figures A.2B and A.2C). For example, these NPCs expressed markers of all A/P regions, including the forebrain (FOXG1 and DLX2), forebrain/midbrain (OTX2), midbrain/hindbrain (EN1 and IRX3), and hindbrain/spinal cord (HOXB4) (Figure A.2E). Moreover, single-cell analysis by FC and immunofluorescence (IF) revealed that our NPC cultures heterogeneously expressed the regionally specific markers FORSE-1 (Figure A.2F; forebrain), PAX6 (Figure A.2G), and HOXB4 (Figure A.2H). Together, these data suggested that these in vitro NPC cultures, as in the developing embryo, are exposed to patterning cues that impart distinct regional identities and neuronal differentiation potentials (Pevny, Sockanathan, Placzek, & Lovell-Badge, 1998; Uwanogho et al., 1995; Wood & Episkopou, 1999). Additionally, we confirmed that these NPCs were able to differentiate to neurons (Figures A.2I–A.2K), including those with a GABAergic identity (Figure A.2J), and glial cells (Figure A.2K).

To investigate the extent to which the high degree of heterogeneity in A/P positional identity correlated with our observed heterogeneity in WNT reporter activity, we performed whole transcriptome RNA sequencing (RNA-seq) on sorted GFP+ and GFP-negative (GFP−) NPCs from passage 5 NPC cultures (Table A.1). Overall, we identified 1,273 genes with statistically significant differential expression between these two cell populations, with expression of 707 genes being elevated in the GFP+ population and 566 genes elevated in the GFP− population

(Figure 2.1C; Table A.1). As expected, WNT target genes such as SP5, LEF1, and AXIN2 were elevated in the GFP+ population (Figure 2.1D; Table A2). Additional analysis of WNT pathway components revealed that expression of the majority of WNT proteins and agonists was higher in GFP+ NPCs, whereas expression of WNT antagonists was higher in GFP− NPCs (Figure 2.1D; Table A.2).

Furthermore, expression of genes with distinct domains of expression along the A/P axis segregated into these two cell populations (Figure 2.1E; Table A.3). Specifically, we found that GFP− NPCs in clone hTOP-19 were enriched for forebrain/anterior-specific markers such as LHX8, DLX2, FOXG1, and LHX2. Conversely, the expression of hindbrain/posterior-related markers such as GBX2, PITX2, FGF8, and IRX3 was increased in GFP+ NPCs. Most notably, members of the HOX gene family, which are highly expressed in the hindbrain and spinal cord, were significantly upregulated in the GFP+ cell population. Finally, various midbrain-associated genes such as EN1, EN2, LMX1A, and LMX1B were expressed in both populations. Collectively, this RNA-seq analysis establishes a correlation between cells that receive a WNT signal (i.e., the GFP+ population) and posterior fates. In contrast, NPCs that do not receive an endogenous WNT signal input (i.e., the GFP− population) are biased toward an anterior identity.

To further characterize the diversity of cells expressing varying levels of GFP in the hTOP-19 NPCs, we used FC to isolate GFP^{HIGH}, GFP^{MID}, and GFP^{LOW} populations (Figure 2.2A). After cell sorting, the specific level of GFP expression remained stable in subsequent culture (Figure 2B). As expected, expression of the WNT target gene AXIN2 was highest in the GFP HIGH cell population and lowest in the GFP^{LOW} NPCs (Figure 2.2C). The three sorted cell populations expressed similar levels of the pan-neural markers SOX1, SOX2, and NESTIN (Figures 2D, S3A, and S3B), demonstrating that NPCs of varying endogenous WNT activity are homogeneous with respect to expression of pan-neural markers.

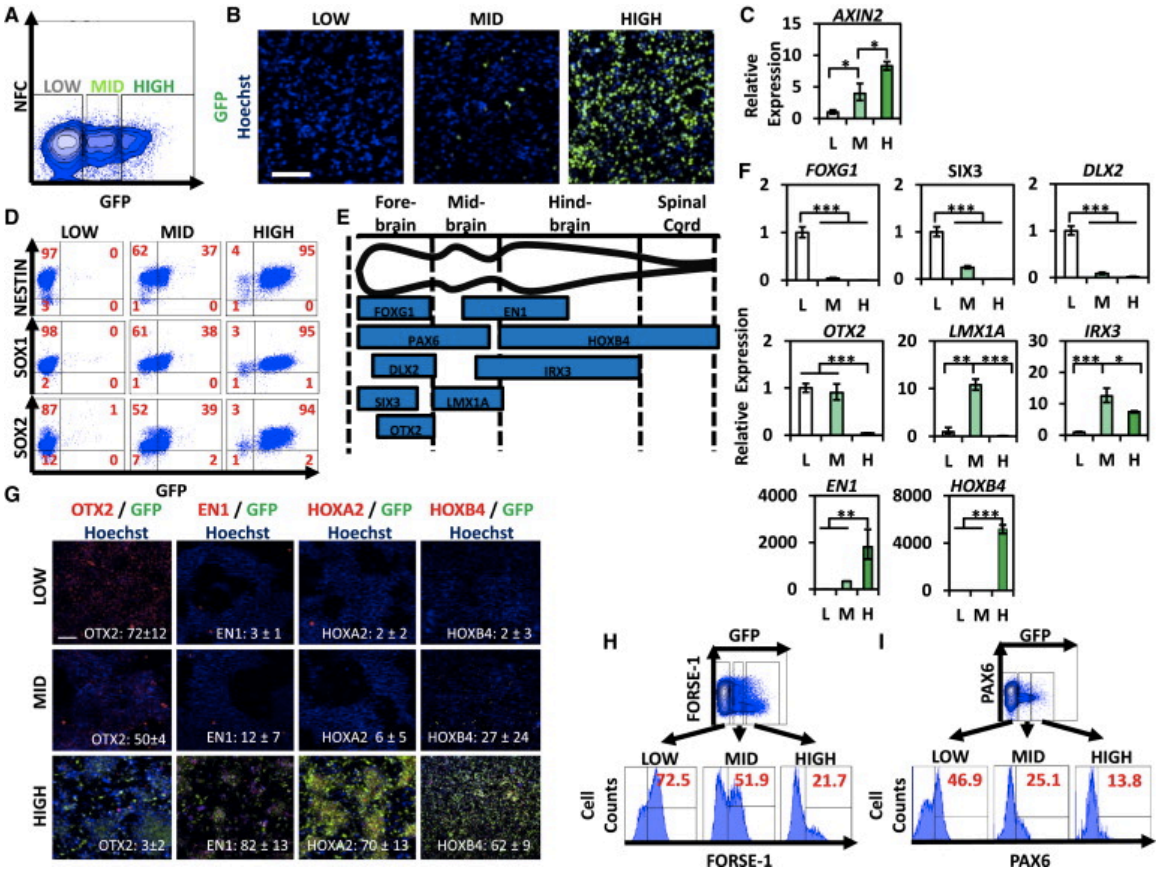

Figure 2.2 Characterization of WNT Reporter Expressing NPC Populations Reveals a Regional Bias A) WNT reporter NPCs were divided into three populations on the basis of GFP expression: GFP^{LOW}, GFP^{MID}, and GFP^{HIGH}. B) Fluorescent images of GFP-sorted NPC populations. GFP expression remains stable after sorting and subsequent culture (scale bar, 100 μm). C) Gene expression of the WNT target gene AXIN2 in GFP-sorted NPC populations (mean ± SEM, n = 3 independent experiments). D) FC of the pan-neural markers NESTIN, SOX1, and SOX2 in GFP-sorted NPC populations. E) Schematic of areas of expression of key genes involved in A/P patterning of the developing neural tube. F) Gene-expression analysis of A/P-related genes in GFP-sorted NPC populations (mean ± SEM, n = 3 independent experiments). Populations were compared using Student's t test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. G) IF of A/P-related markers in GFP expression in GFP^{LOW}, GFP^{MID}, and GFP^{HIGH} (scale bar, 100 μm). H and I) FORSE-1 and (I) PAX6 expression in unsorted and sorted GFP-expressing NPC populations. Isotype controls used are listed in Table S5. NFC, nonfluorescing channel.

We examined the A/P expression profile (Figure 2.2E) of NPCs with different levels of endogenous WNT signaling by quantitative PCR (qPCR; Figure 2F), IF (Figure 2.2G), and FC (Figures 2.2H and 2.2I). Expression of the forebrain markers FOXG1, SIX3, and DLX2 was highest in GFP^{LOW} NPCs. Expression of OTX2, which is expressed at the forebrain-midbrain boundary, was similar in the GFP^{LOW} and GFP^{MID} populations but absent in the GFP^{HIGH} NPCs. Expression of the midbrain marker LMX1A was highest in the GFP^{MID} population. The midbrain/hindbrain boundary markers IRX3 and EN1 were expressed at high levels in both the GFP^{MID}- and GFP^{HIGH}-sorted cell populations. The hindbrain markers HOXA2 and HOXB4 were highly expressed in the GFP^{HIGH} NPCs and diminished in both the GFP^{MID} and GFP^{LOW} populations. Finally, we performed FC on unsorted GFP-expressing hTOP-19 NPCs. This analysis demonstrated a negative correlation between GFP levels and expression of the anterior neural cell-surface marker FORSE-1 (Figure 2H). (Elkabetz et al., 2008) PAX6, which is expressed in the diencephalon and midbrain during early development, was expressed primarily in the GFP^{LOW} and GFP^{MID} cell fractions (Figure 2I). Together, these data suggest that the level of endogenous WNT signaling correlates with the positional identity of hESC-derived NPCs.

To determine whether the effect of endogenous WNT signaling on the regional patterning of NPCs was stable, we cultured sorted GFP^{HIGH} and GFP^{LOW} populations for ten passages (>50 days) and examined the expression of A/P-related genes (Figure S3C). Expression of AXIN2 remained stable in the GFP^{HIGH} NPCs, and AXIN2 expression did not increase in the GFP^{LOW} NPCs over ten passages (Figure S3D). During the course of ten passages, the forebrain markers FOXG1, SIX3, and OTX2 were expressed in the GFP^{LOW} NPCs, but not in the GFP^{HIGH} NPCs (Figure S3E). Conversely, the hindbrain-associated genes IRX3, EN1, and HOXB4 were stably expressed in the GFP^{HIGH} NPCs, but were not elevated in the GFP^{LOW} NPCs during the period of ten passages (Figure S3E). In sum, these data indicate that the positional identity of NPCs is stable during long-term culture.

2.3.2. The level of endogenous WNT activity instructs the neuronal differentiation potential

We wanted to determine whether the level of endogenous WNT signaling present in NPCs conferred a regional bias as they were expanded and subsequently differentiated to neurons in vitro. To address this issue, FC-purified GFP^{HIGH}, GFP^{MID}, and GFP^{LOW} hTOP-19 NPCs were expanded for four passages and then differentiated to neurons (Figure 3A). After 4 weeks of differentiation, all three cell populations generated cells with a neuronal morphology (Figure S4A). Additionally, IF demonstrated that each NPC population yielded similar numbers of MAP2+ and B3T+ neurons (Figures A.4B and A.4C). Importantly, gene expression (Figures 2.3B–2.3D) and IF analysis (Figure 2.3E) of neurons generated from these different WNT reporter NPC populations revealed distinct regional identities. Neurons generated from GFP^{LOW} NPCs expressed the highest levels of FOXG1 (a marker of neurons with telencephalic identity), SATB2 (labels cortical neurons of layers II/III), CTIP2 (expressed by striatal medium spiny neurons), EMX1 (a marker for pyramidal neurons of the cerebral cortex), CUX1 (expressed in layer IV-II late-born/upper-layer cortical neurons), and TBR1 (labels cortical neurons, especially those associated with layer VI). By comparison, GFP^{MID} NPCs differentiated into neurons with a midbrain phenotype, including expression of the midbrain GABAergic-associated marker GATA3 and the midbrain dopaminergic (mDA)-related markers LMX1A/1B (regulate mDA progenitor proliferation, specification, and differentiation), NURR1 (specifies neurotransmitter identity of mDA neurons), PITX3 (regulates tyrosine hydroxylase [TH] expression in mDA neurons), and TH (the enzyme responsible for generation of L-DOPA, which is a precursor for the neurotransmitter dopamine). Finally, neurons differentiated from GFP^{HIGH} NPCs expressed the highest levels of hindbrain/spinal-cord-associated genes, such as HOXA2, HOXB4, and HOXB6, as well as the motor neuron marker MNX1 (also known as HB9).

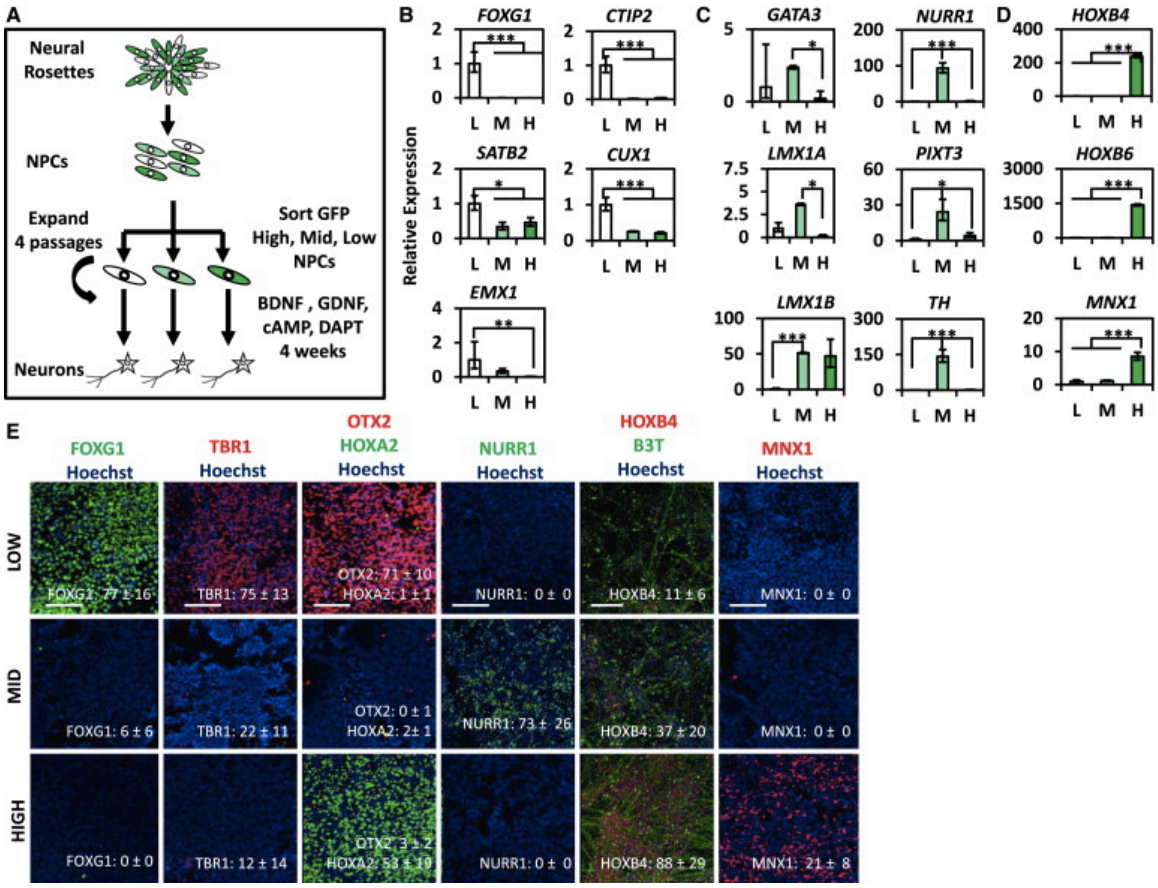

Figure 2.3 Neuronal Differentiation Bias of Sorted GFP-Expressing NPC Populations A) Schematic of the experimental protocol. FC was used to sort GFP-expressing NPCs into GFP^{LOW}, GFP^{MID}, and GFP^{HIGH} populations. The sorted cell populations were expanded for four passages and then differentiated to neurons. B-D) Gene-expression analysis of (B) cortical/forebrain-, (C) midbrain-, and (D) hindbrain/spinal-cord-related neuronal genes in neurons derived from sorted GFP-expressing NPC populations (mean ± SEM, n = 4 independent experiments). Populations were compared using Student's t test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. E) IF of cortical/forebrain-, midbrain-, and hindbrain/spinal-cord-associated markers in neuronal cultures differentiated from sorted GFP-expressing NPC populations (scale bar, 100 μm). L, GFP^{LOW}; M, GFP^{MID}; H, GFP^{HIGH}.

2.3.3. Exogenous modulation of WNT signaling influences NPC positional identity and reduces heterogeneity in neuronal differentiation

The above analysis demonstrates that endogenous WNT signaling activity correlates with A/P regional identity of NPCs. To determine the extent to which WNT signaling is instructive in conferring regional bias, we used several methods to perturb WNT signaling during NPC generation. Specifically, we differentiated four independent hPSC lines (H9, HUES9, HES3, and RiPSC; (Warren et al., 2010)) to NPCs while activating or inhibiting WNT signaling with CHIR 98104 (CHIR, a potent inhibitor of GSK-3β) and IWP2, respectively (Figure 2.4A). Activation of WNT signaling with CHIR treatment led to an increase in embryoid body (EB) size, whereas inhibition of endogenous WNT signaling through IWP2 treatment resulted in a decrease in EB size (Figures A.5A and A.5B), consistent with the known role of canonical WNT signaling in promoting proliferation in hESC-derived neurospheres (Davidson et al., 2007). CHIR-treated and IWP2-treated NPCs expressed levels of the pan-neural markers SOX1, SOX2, and NESTIN similar to those observed for untreated NPCs (Figures A.5C–A.5E).

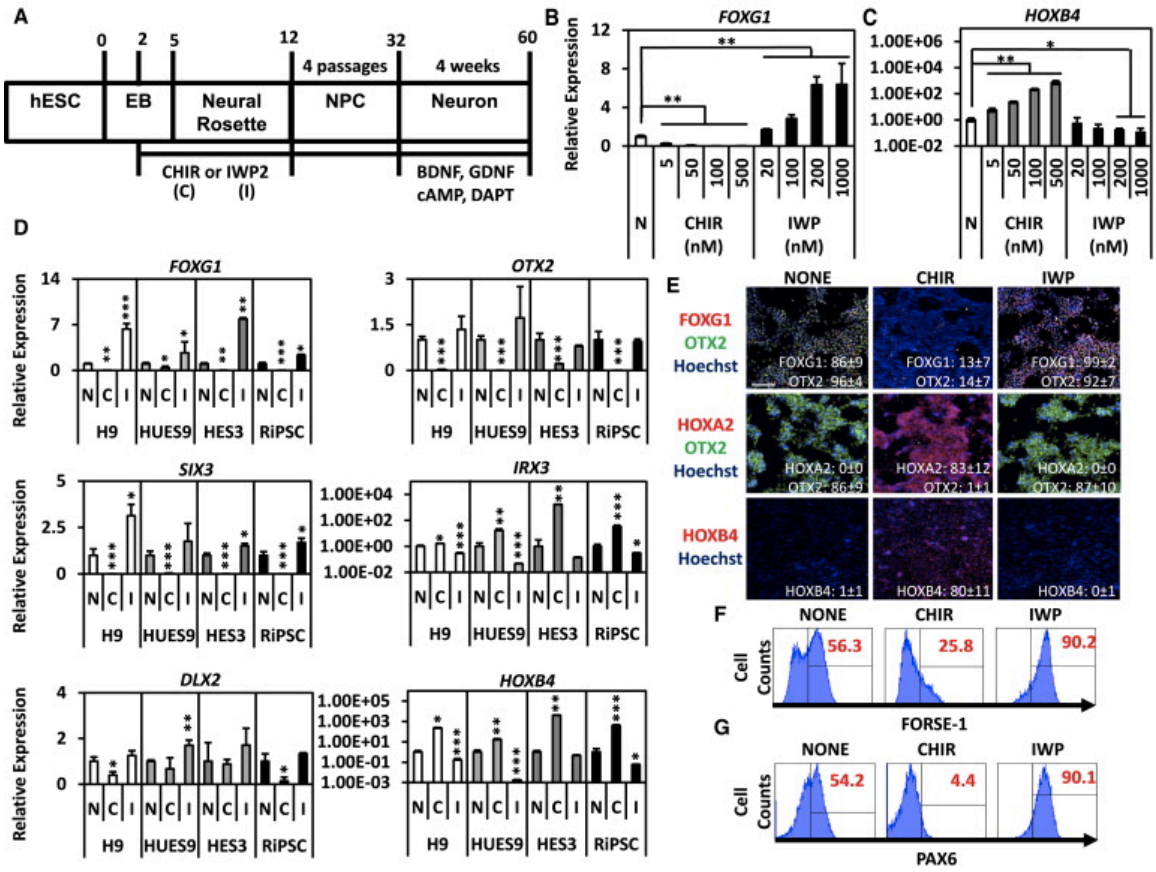

Figure 2.4 Exogenous Manipulation of WNT Signaling Reduces Heterogeneity in NPC Cultures

A) Outline of the experimental protocol. CHIR or IWP2 was added during day 2 of neural differentiation. CHIR- and IWP2-treated and untreated NPCs were expanded for four passages prior to differentiation to neurons. B and C) Gene-expression analysis of (B) FOXG1 and (C) HOXB4 in NPC cultures derived from H9 hPSCs in the presence of varying levels of CHIR and IWP2. D) /P gene expression in 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures derived from H9, HUES9, HES3, and RiPSC hPSCs (mean ± SEM, n = 3 independent experiments). Populations were compared with untreated (N) cells using Student's t test. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001. E) IF of A/P-related markers in 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures (scale bar, 100 μm). F and G) FC of (F) FORSE-1 and (G) PAX6 in 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures. Isotype controls used are listed in Table S5. N, none; C, CHIR 98014; I, IWP2.

Although there was no effect of WNT signal perturbations on the formation of SOX1+, SOX2+, and NESTIN+ NPCs, CHIR or IWP2 treatment influenced the A/P expression profile of NPCs. Expression of the anterior marker FOXG1 decreased in a dose-dependent manner with CHIR treatment, but increased with IWP2 treatment (Figure 2.4B). Conversely, the levels of the posterior marker HOXB4 increased in a concentration-dependent manner with CHIR treatment, but decreased with IWP2 treatment (Figure 2.4C). The expression of A/P markers was also influenced by CHIR and IWP2 treatment across all hPSC lines tested (Figures 2.4D–2.4G). Specifically, expression of the anterior markers FOXG1, FORSE-1, SIX3, DLX2, OTX2, and PAX6 was higher in IWP2-treated cells, whereas expression of the posterior markers IRX3, HOXA2, and HOXB4 was higher in CHIR-treated cells. To eliminate the possibility that the posteriorizing effect of CHIR was not due to the activation of other signaling pathways that act through GSK3 (Jope & Johnson, 2004), we also generated NPCs in the presence of WNT3a (K. H. Willert, 2008). Similar to the effect of CHIR on A/P gene expression of NPCs, addition of WNT3a during NPC formation led to an increase in expression of the posterior genes LMX1A, EN1, IRX3, and HOXB4, and a decrease in expression of the anterior genes FOXG1, SIX3, DLX2, and OTX2 (Figure A.5F). These data demonstrate that exogenous activation or inhibition of canonical WNT signaling can be used to control the positional patterning of NPCs in differentiating hPSCs. Additionally, the observation that inhibition of endogenous WNT signaling with IWP2 during NPC generation reduced the expression of genes associated with posterior identity suggests that endogenous WNT signaling specifies a posterior identity, consistent with its known function in CNS development.

To examine whether patterning of NPCs imposed by exogenous WNT manipulation was stable, we cultured CHIR- and IWP2-treated NPCs in the absence of these exogenous signals for ten passages (>50 days) (Figures A.6A and A.6B). After ten passages, the expression level of the forebrain marker FOXG1 remained unchanged in NPCs that were generated in the presence of IWP2 (Figure A.6C). Along similar lines, the expression level of the hindbrain marker HOXB4 remained constant over ten passages of the NPCs that were generated in the presence of CHIR (Figure A.6D). Therefore, continued WNT pathway modulation during prolonged culture was not

required to maintain the regional identity of NPCs. Furthermore, we examined the ability of NPCs to alter their positional identity after NPC formation. To test this, we treated regionally specified NPCs with CHIR or IWP2 for ten passages after NPC formation (Figures A.6A and A.6B). Addition of CHIR to anterior-specified NPCs (i.e., NPCs formed in the presence of IWP2) had no effect on FOXG1 or HOXB4 expression (Figures A.6C and A.6D). Likewise, IWP2 treatment of posterior-patterned NPCs (i.e., NPCs formed in the presence of CHIR) did not change their regional identity (Figures A.6C and A.6D). Collectively, these data suggest that the effect of exogenous WNT signaling on NPC patterning is imparted early during their generation and NPCs are unable to interconvert between positional identities during subsequent expansion.

We subsequently investigated whether NPCs generated with IWP2 or CHIR treatment retained their regional phenotype upon differentiation to neurons. IWP2- and CHIR-treated NPCs were expanded for four passages and then subjected to the neuronal differentiation protocol. IF (Figure 2.5A) and gene expression (Figure A.5G) revealed that IWP2- and CHIR-treated NPCs generated similar numbers of B3T+ neurons compared with untreated NPCs. However, neurons generated from IWP2-treated NPCs expressed higher levels of the forebrain- and cortical-related neuronal markers FOXG1, TBR1, SATB2, CTIP2, EMX1, CUX1, and OTX2 compared with neurons generated from CHIR-treated or -untreated NPCs (Figures 2.5B and 2.5C). On the other hand, neurons generated from CHIR-treated NPCs expressed higher levels of the hindbrain- and spinal-cord-specific markers HOXA2, HOXB4, HOXB6, and MNX1 (Figures 2.5B and 2.5C). Together, our results indicate that NPCs retain their regional identity over multiple passages and manifest that identity in differentiated neuron cultures.

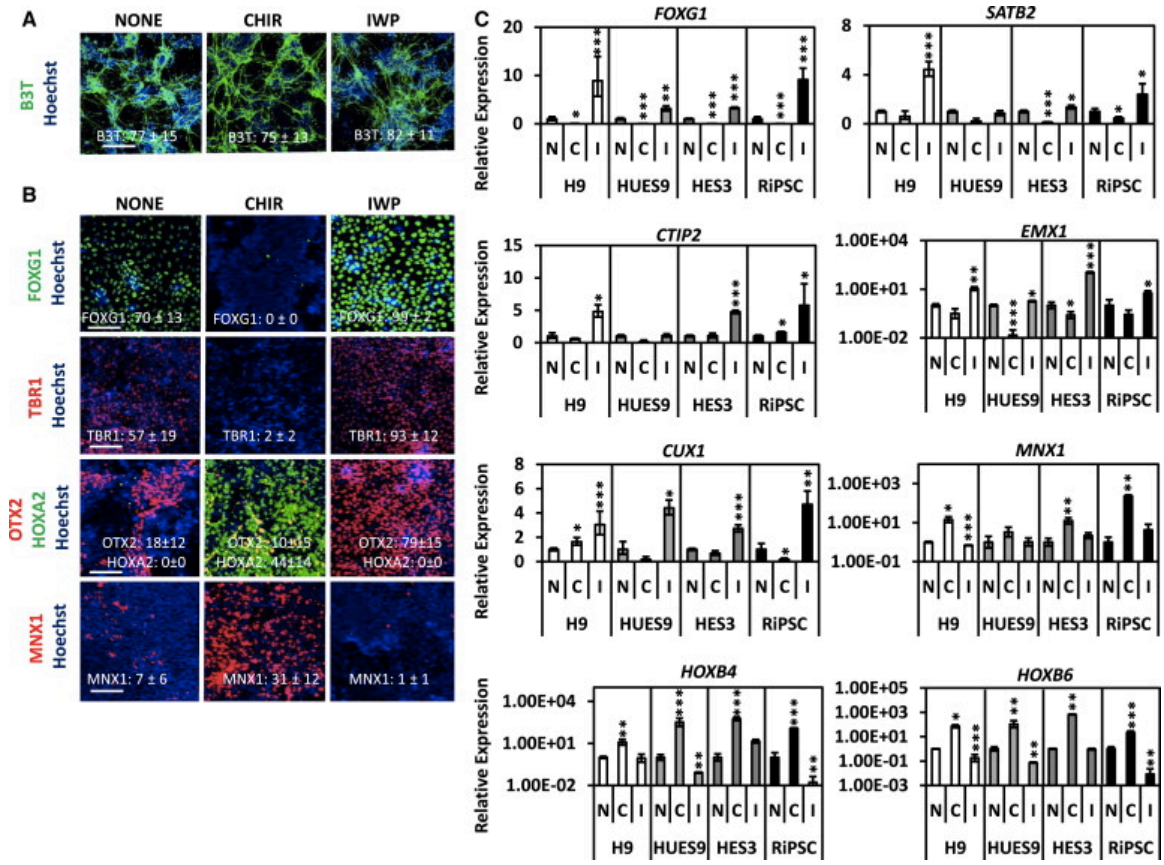

Figure 2.5 Analysis of Neurons Derived from CHIR- and IWP2-Treated and Untreated NPC Cultures A) IF of mature neuronal markers B3T in neuronal cultures differentiated from 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures derived from H9 hPSCs (scale bar, 100 μm). B) IF of cortical-, forebrain-, midbrain-, hindbrain-, and spinal-cord-related neuronal genes in neurons differentiated from 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures derived from H9 hPSCs (scale bar, 100 μm). C) Expression of cortical-, forebrain-, midbrain-, hindbrain-, and spinal-cord-related neuronal genes in neurons differentiated from 500 nM CHIR- and 1,000 nM IWP2-treated and untreated NPC cultures derived from H9, HUES9, HES3, and RiPSC hPSCs (mean ± SEM, n = 4 independent experiments). Populations were compared with neurons differentiated from untreated (N) NPCs using Student's t test. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001.

Although we were able to generate NPC and neuron cultures with distinct anterior and posterior identities, we did not observe cell populations expressing markers associated with a midbrain phenotype. We speculated that this notable absence of midbrain cell types may have

been the consequence of endogenous WNT signaling activities that influence the extent to which exogenous WNT signaling imparts A/P identities. To eliminate the contribution of any endogenous WNT signaling during NPC generation and fine-tune the level of WNT signaling activity, we treated differentiating cultures (hPSCs to NPCs) with IWP2 simultaneously with increasing concentrations of CHIR. Analysis of the gene-expression profile of NPCs derived at various concentrations of CHIR revealed that we could specify the A/P positional identity of NPCs by precisely controlling the level of exogenous WNT signaling (Figures 2.6A–2.6C). In all hPSC lines tested, induction of NPCs with a midbrain phenotype occurred at a narrow CHIR concentration range, with the precise CHIR range varying slightly between hPSC lines. CHIR concentrations significantly lower or higher than these optima led to the production of NPCs with an anterior/forebrain or hindbrain/spinal-cord phenotype, respectively.

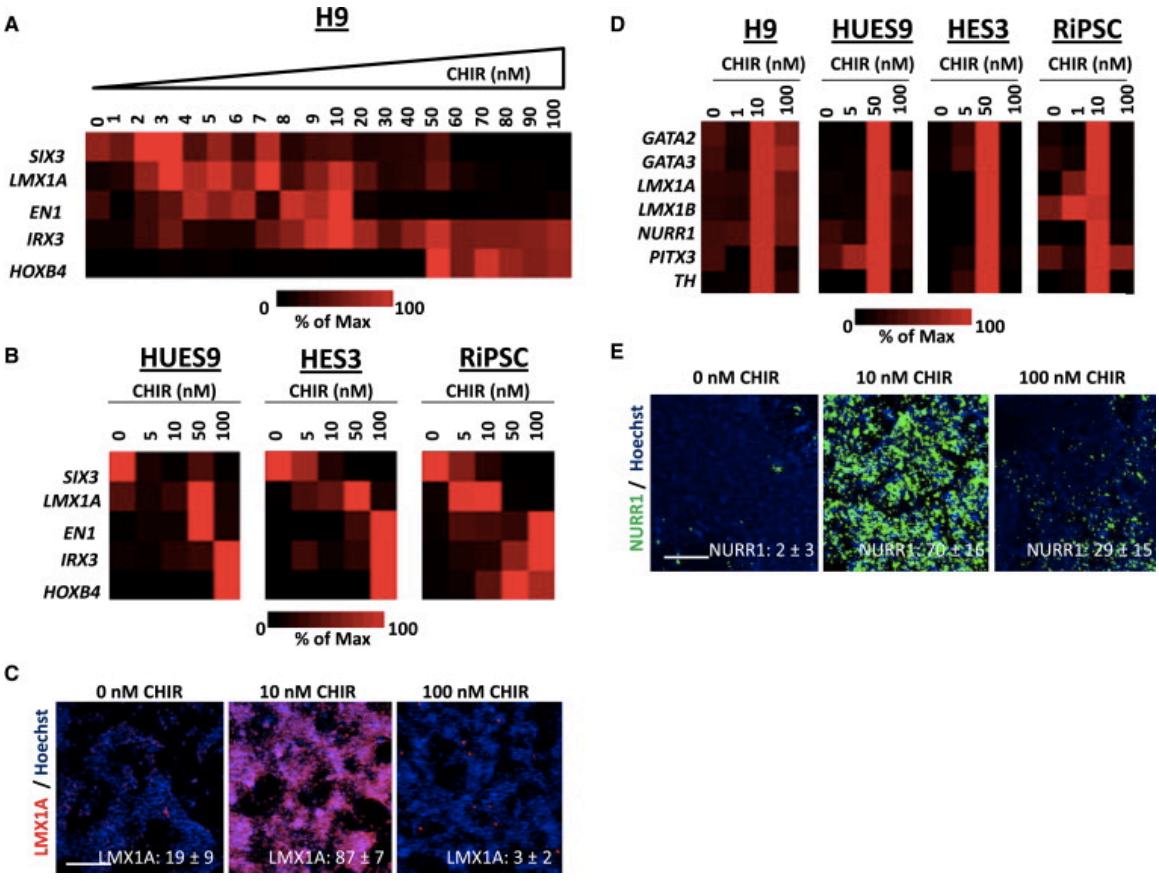

Figure 2.6 Specification of the Midbrain Neural Phenotype through Precise Exogenous Manipulation of WNT Signaling A and B) Gene-expression analysis of A/P-related genes in NPCs

generated from (A) H9 (mean, n = 3 technical replicates) and (B) HUES9, HES3, and RiPSC (mean, n = 3 independent experiments) hPSCs in the presence of 1,000 nM IWP2 and various concentrations of CHIR. The data are displayed in a heatmap where black corresponds to minimum expression levels and red corresponds to maximum levels. For each gene analyzed, the expression levels were normalized to the sample with the highest expression level. C) IF of LMX1A expression in NPCs generated from H9 hPSCs in the in the presence of 1,000 nM IWP2 and various concentrations of CHIR (scale bar, 100 μm). D) Gene-expression analysis of midbrain-related genes in neuronal cultures differentiated from NPCs treated with various CHIR concentrations (mean; H9, n = 3 independent experiments; HUES9, HES3, RiPSC, n = 4 independent experiments). E) IF of NURR1 expression in NPCs generated from H9 hPSCs in the in the presence of 1,000 nM IWP2 and various concentrations of CHIR (scale bar, 100 μm).

We differentiated these NPCs of specific positional fate to neurons. Expression of the midbrain neuronal-related markers GATA2, GATA3, LMX1A, LMX1B, NURR1, PITX3, and TH peaked at a CHIR concentration of 10 nM for NPCs generated from H9 or RiPSC hPSCs, and a CHIR concentration of 50 nM for NPCs generated from HUES9 or HES3 hPSCs (Figures 2.6D and 2.6E). Together, these data indicate that by selecting the precise level of extrinsic WNT signaling in the context of suppressed endogenous WNT signaling, we could generate hPSC-derived NPCs and neurons with midbrain characteristics.

2.4. Conclusion

hPSCs and their derivative NPCs share the ability to self-renew indefinitely while retaining the potential to differentiate into mature cell types, although the potential of NPCs is restricted to cells of the CNS, including neurons, astrocytes, and oligodendrocytes. Our study demonstrates that NPCs are additionally restricted by their positional identity. As in development of the CNS, where establishment of the A/P axis early during neural tube formation confers a specific positional identity on naive neuroepithelial cells, NPCs acquire similar positional information from their microenvironment. Importantly, despite their apparent homogeneous appearance and lack of a clear 3D architecture, NPCs in cell culture acquire and stably maintain this A/P positional identity. In contrast to this stable identity of NPCs, where individual and heterogeneous states

coexist and do not interconvert, ESCs exist in a metastable state in which individual cells exhibit oscillatory expression of transcription factors (Cahan & Daley, 2013; Canham, Sharov, Ko, & Brickman, 2010; de Souza, 2012; Galvin-Burgess, Travis, Pierson, & Vivian, 2013; Narsinh et al., 2011; Stewart et al., 2006), correlating with a bias to either self-renew or differentiate. Importantly, we show that NPC positional identity and specific neuronal differentiation potential are retained over prolonged expansion (>50 days) in culture.

The WNT signaling pathway is a primary determinant in assigning an A/P positional identity to NPCs. This instructional cue is imparted early during NPC generation and once this identity is established, it is stable and cannot be altered through exogenous manipulation of the WNT pathway. Using a WNT reporter line, we show that endogenous WNT signaling is highly variable among individual cells as they acquire a NPC phenotype, with cells of posterior identity expressing WNT reporter activity. In addition to expressing markers of posteriorly fated NPCs, most notably genes of the HOX gene cluster, these cells also express multiple WNT ligands. In contrast, NPCs with anterior identity, as detected by a lack of WNT reporter activity, express multiple WNT antagonists. These differences in expression of WNT agonist and antagonist resemble those observed in the developing neural tube in vivo, with posterior tissues expressing WNT proteins and anterior tissues expressing WNT antagonists such as DKK1 and FRZB (Hashimoto et al., 2000; Leyns et al., 1997).

These opposing WNT signals generate an endogenous gradient of WNT activity, which divides the embryonic neural tube along the A/P axis into distinct progenitor domains, each of which gives rise to specific regionalized neurons (Ciani & Salinas, 2005; Kiecker & Niehrs, 2001; Nordström, Jessell, & Edlund, 2002). These progenitor domains have regionally specific gene-expression profiles and differentiation predispositions despite similar levels of expression of the pan-neural markers SOX1 and SOX2 (Pevny et al., 1998; Wood & Episkopou, 1999; Zappone et al., 2000). Here, we showed that NPCs exhibited a broad range of endogenous WNT activity that conferred specific regionalized fates despite comparable expression levels of SOX1 and SOX2, perhaps mimicking the same developmental events that are seen during early in vivo neural tube

development. Therefore, the local WNT microenvironment tightly regulates the WNT activity status and hence the positional identity of NPCs.

A somewhat unexpected implication of these gene-expression patterns is that WNT signaling appears to be acting cell autonomously, with WNT signaling activity restricted to those cells expressing WNT genes. Although WNT signaling activity is present in a graded fashion in these NPC cultures, WNT proteins are acting in an autocrine rather than paracrine manner. Furthermore, expression of WNT antagonists may mute the response in cells near or adjacent to WNT secreting cells. A more careful analysis of this cell-based system will likely yield important mechanistic insights into the dynamic nature of WNT signaling during development.

This restricted WNT signaling activity observed in NPC cultures is consistent with the notion that WNT proteins act locally (Habib et al., 2013) and exhibit minimal, if any, extracellular diffusion. A recent study demonstrated that flies expressing an engineered membrane-tethered Wingless (a fly WNT protein) are viable and normally patterned, suggesting that the spread of Wingless is dispensable for patterning and growth (Alexandre et al., 2013). Similarly, in our cell-based system, WNT proteins act locally and do not signal to distant cells. In addition, expression of WNT antagonists in the WNT− populations may act to block paracrine WNT signaling activity. This local WNT activity is not the result of the physical separation of distinct WNT expressing domains, since this localized activity is retained in a mixed and seemingly homogeneous cell culture system.

While endogenous WNT signaling activity is a major source of heterogeneity among individual NPCs, exogenous manipulation of this signaling pathway can be exploited to impart specific positional identities to NPCs during their generation from hPSCs, thereby reducing cellular heterogeneity. Activation of WNT signaling with purified WNT3a protein or a GSK-3β inhibitor (CHIR98014) led to the generation of NPCs with a hindbrain/spinal cord identity, whereas inhibition of WNT signaling with a PORCN inhibitor (IWP2) to block endogenous WNT protein processing led to the generation of NPCs with a forebrain phenotype. As shown in this and other studies (X.-J. Li et al., 2009; Pankratz et al., 2007), in the absence of any WNT pathway manipulations, NPCs generated form hPSCs are generally biased toward an anterior

fate, suggesting that endogenous WNT signaling in these culture systems is relatively low and insufficient to promote posterior fates. Consequently, ectopic activation of WNT signaling produces a prominent shift from an anterior to a posterior fate. In contrast, in the absence of WNT signaling (through IWP2 addition), the relative increase of anterior-related markers, though statistically significant, is less pronounced.

Although exogenous manipulation of WNT signaling can be used to reduce NPC heterogeneity, the window during which this manipulation is effective is limited. Specifically, we find that WNT signaling imparts positional identity early during NPC generation, likely during the rosette stage, in which the cell population most closely resembles the early developing neural tube. Once an NPC culture is established and propagated over multiple passages, A/P positional identity is stable and recalcitrant to exogenous manipulations of WNT signaling. Therefore, the identity, concentration, and timing of factors (e.g., WNTs) added during NPC generation are critical to produce homogeneous cultures.

Although WNT signaling plays a prominent role in A/P patterning of the neural tube, few studies have extensively examined the influence of WNT on the A/P positional identity of hPSC-derived NPCs and neurons. To date, most studies have relied on FGF8 (Y. Yan et al., 2005; D. Yang, Zhang, Oldenburg, Ayala, & Zhang, 2008) or retinoic acid (RA) (Dimos et al., 2008; Hu & Zhang, 2009; X.-J. Li et al., 2005, 2008; Singh Roy et al., 2005) to generate posterior neural populations, such as midbrain dopaminergic and spinal cord motor neurons, respectively, from hPSCs. Similar to the approach we used in our study, two groups recently used a specific concentration of the GSK3β inhibitor CHIR99021 (an analog to the GSK3β inhibitor used in this study) to generate midbrain dopaminergic neurons from hPSCs (Kirkeby, Grealish, et al., 2012a; Kriks et al., 2011). However, the ability of this compound to generate stable NPC populations from different areas of A/P axis was not extensively studied. We demonstrated that through precise chemical modulation of WNT signaling, we could control the A/P positional identity of hPSC-derived NPCs. Moreover, we demonstrated that these NPCs retained their positional specificity as they were differentiated to neurons in vitro. It should also be noted that previous studies (Hu & Zhang, 2009; Y. Yan et al., 2005) have relied on the activation of SHH signaling in

order to generate ventral neurons, such as mDA and motor neurons. In this study, we demonstrated that we were able to generate these ventral neuronal subtypes without exogenous modulation of SHH signaling. This suggests the possibility that endogenous SHH signaling may regulate the dorsal-ventral (D/V) identity of hPSC-derived NPCs and neurons analogously to the manner in which endogenous WNT signaling regulates their A/P identity. Nonetheless, our study serves as proof-of-principle that modulation of developmental signaling pathways, such as WNT and SHH, can be exploited to refine the A/P and D/V identity of NPCs and neurons.

Our findings regarding the positional restriction of NPCs have important implications for the study and application of these cells. Several studies have described the transplantation of hPSC-derived NPCs into animal models of Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and spinal cord injury (S. U. Kim, Lee, & Kim, 2013; Lindvall & Kokaia, 2010). However, few of these studies have described a successful long-term reduction in the symptoms associated with these disorders (S. U. Kim et al., 2013; Lindvall & Kokaia, 2010). Interestingly, several of these studies relied on NPCs generated by dual SMAD inhibition, which results in NPCs of an anterior telencephalic identity (Chambers et al., 2009), which may explain the lack of symptomatic improvement in animal models of disorders associated with the midbrain, hindbrain, and spinal cord. We speculate that future animal transplantation studies that utilize regionally specific and stably expandable NPCs, such as those generated in this study, will result in a significant improvement in the cognitive and motor deficits associated with these neurological disorders.

In summary, we determined that endogenous WNT signaling influences the heterogeneity, regional characteristics, and differential potential of hPSC-derived NPCs. In addition, we showed that precise exogenous modulation of WNT signaling during neural differentiation of hPSCs results in a homogeneous NPC population with a specific positional identity. Importantly, manipulation of endogenous and exogenous WNT signaling will allow for the development of defined methods for generating transplantable hPSC-derived NPCs for specific regions of the entire A/P axis of the neural tube. Furthermore, this study suggests that modulation of other developmental signaling pathways, including BMP, FGF, and SHH, can be exploited to further

refine the positional identity of NPCs and neurons. In the future, these regionally specific NPCs will greatly enhance the translational potential of hPSCs for neural-related therapies.

CHAPTER 3

TRANSCRIPTIONAL REGULATION OF NEURAL FATE THROUGH A MULTI-STATE MODEL OF WNT/B-CATENIN SIGNALING

3.1. Introduction

Multiple coordinating signaling pathways are required for proper induction and patterning of the vertebrate neural tube. This process is hypothesized to follow a two-step process, neural induction or “activation” which entails acquisition of an anterior fate followed by progressive caudalization with a “transforming” factor (Nieuwkoop, 1952; Nieuwkoop & Nigtevecht, 1954). Neural induction is well known to rely on the action of the Spemann organizer, discovered through groundbreaking transplantation experiments, which emits signals to antagonize BMP signaling, this serves as the “activation” step (Hemmati-Brivanlou et al., 1994; SASAI, 1994; Sasai et al., 1995; Smith & Harland, 1992). The transformative factor remained elusive for many years and was hypothesized to be due to the action of morphogens such as WNTs, FGFs, or RA.

WNTs compose a large family of evolutionarily conserved lipid-modified, secreted glycoproteins (Clevers & Nusse, 2012). WNT signaling plays a diverse set of roles and influences many cell processes fundamentally related to symmetry breaking, these processes can broadly be divided into controlling ‘cell fate’ (canonical) or ‘cell polarity’ (non-canonical) (Loh, van Amerongen, et al., 2016). Canonical WNT signaling hereafter referred to as WNT/β-catenin signaling, is better studied than non-canonical WNT signaling and focuses on the fate of β-catenin. In the absence of a WNT ligand, a complex of proteins known as the destruction complex constitutively degrades β-catenin (Jennifer L Stamos & Weis, 2013). WNT bound to its cognate frizzled (Fz) receptor triggers formation of disheveled (DVL) dependent LRP6 signalosomes at the plasma membrane, which subsequently recruits Axin, thus dissociating the destruction complex and allowing accumulation of β-catenin in the cytoplasm (Bilic et al., 2007) β-catenin then translocates to the nucleus where is recruited to chromatin via transcription factors (TFs), most well characterized are the TCF/LEF TFs, to bind chromatin and activate WNT target gene expression (Cadigan, 2012) As a function of its diverse cellular roles, aberrant WNT signaling

results in a variety of severe developmental disorders and multiple forms of cancer (Clevers & Nusse, 2012; Logan & Nusse, 2004).

WNT/β-catenin plays an essential role as a morphogen in patterning the anteroposterior axis in the developing neural tube (Kiecker & Niehrs, 2001; McGrew et al., 1995; Nordström et al., 2002) A progressive gradient establishes more caudal neural fates. Local inhibition of WNT signaling in the caudal region antagonizes WNT signaling in the prospective telencephalon to ensure proper development (Glinka, Wu, Onichtchouk, Blumenstock, & Niehrs, 1997). When WNT inhibition is prevented such as through mutation in TCF3 (C. H. Kim et al., 2000) or inhibition of DKK1/SFRPs, the telencephalon fails to develop, leading to expansion of caudal fates. (Mukhopadhyay et al., 2001) Conversely, WNT3a mutant mice or introduction of a dominant negative xWNT8 result in severe abnormalities in hindbrain and spinal cord patterning (Erter, Wilm, Basler, Wright, & Solnica-Krezel, 2001; Lekven, Thorpe, Waxman, & Moon, 2001; Takada et al., 1994). Additionally WNTs are thought to act in concert with other morphogens such as FGFs and RA to ensure proper patterning of the developing neural tube (Gavalas & Krumlauf, 2000; Hikasa & Sokol, 2013; Holowacz & Sokol, 1999; Kudoh, Wilson, & Dawid, 2002; McGrew et al., 1997; Partanen, 2007). Multiple labs, including ours have leveraged this information to generate regionally patterned hNPCs and organoids from human pluripotent stem cells (hPCSs) for disease modeling and cell transplantation (Di Lullo & Kriegstein, 2017; Kirkeby et al., 2012; Moya, Cutts, Gaasterland, Willert, & Brafman, 2014).

Although many studies have been performed to characterize the complex gene regulatory networks responsible for patterning and WNT/ β-catenin has well characterized activity in neural patterning the transcriptional mechanisms due to WNT/β-catenin signaling in the developing neural tube are not well understood. Here we utilize a protocol previously established in the lab to generate patterned hNPCs in conjunction with β-catenin chromatin immunoprecipitation sequencing (CHIP-seq) to understand the transcriptional events of β-catenin, which lead to proper neural patterning (Cutts, Brookhouser, & Brafman, 2016; Moya et al., 2014). We find that different levels of WNT signaling lead to mainly unique β-catenin binding. Additionally, identification of enhancers in patterned hNPCs revealed that β-catenin binds many of these cis-

regulatory elements, consistent with a view of β-catenin binding to promote enhancer-promoter looping to regulate transcription. (Estarás et al., 2015; Yochum, Sherrick, Macpartlin, & Goodman, 2010) This study provides mechanistic insight into how graded levels of β-catenin regulate cell fate in neural patterning in the developing neural tube.

3.2. Experimental Methods

3.2.1. Human embryonic stem cell culture (hESC)

hPSCs were passaged in feeder free conditions in Essential 8 medium (Life Technologies) on Matrigel (BD Biosciences). Cells were routinely passaged with Accutase (Millipore) and replated at a density of 4.25 x 10^4 /cm^2 every 3-4 days.

3.2.2. Human neural progenitor cell (hNPC) generation

Human pluripotent stem cell (hPSC)-derived neural progenitor cells (hNPCs) were generated as previously described. (Cutts et al., 2016) Briefly hESCs were dissociated to single cell using Accutase (Stem Cell Technologies) for 5 min and resuspended in neural induction media (1% N2/1% B27 without vitamin A/DMEM:F12) supplemented with 5 uM Y-26732 (Santa Cruz Biotechnology), 50 ng/ml recombinant human noggin (R&D Systems), and 0.5 µm Dorsomorphin (Santa Cruz Biotechnology). Next, 2*10^6 cells were pipetted to each well of a 6-well ultra low-attachment plate (Greiner Bio-One) and placed on an orbital shaker at 95 rpm in a 37 ^oC/5% CO_2 tissue culture incubator. The next day, cells formed spherical clusters (embryoid bodies (EBs)) and a half media change was performed. After 2 days in culture IWP2 (Sigma-Aldrich) or CHIR98014 (Sigma-Aldrich) were added. Media changes were subsequently performed every day.

3.2.3. Quantitative PCR (qPCR)

RNA was isolated from cells using the NuceloSpin® RNA kit (Macherey Nagel). Reverse transcription was performed with iScript RT Supermix (Bio-Rad). Quantitative PCR was carried out using SYBR green dye on a CFX384 Touch™ Real-Time PCR Detection System. QPCR experiments run with SYBR green dye were carried out using iTaq Universal SYBR Green Supermix (Bio-Rad). For qPCR experiments run with SYBR green dye, a 2 min gradient to 95 ^oC followed by 40 cycles at 95 ^oC for 5 s and 60 ^oC for 30 s was used. The list of primer sequences

used in provided in Supplementary Table 1. Gene expression was normalized to 18S rRNA levels. Delta Ct values were calculated as $Ct^{target} – Ct^{18s}$. Relative fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. Data are presented as the average of the biological replicates ± standard error of the mean (S.E.M.).

3.2.4. Generation of CTNNB1-3xFLAG hESC line

Cas9 protein was purchased from PNA Bio Inc (Cat.No. CP01). The full length of gRNA carrying 20 mer target sequence of the human beta-catenin gene (ttacctaaaggatgatttac) was In Vitro synthesized by using HiScribe T7 High Yield RNA Synthesis Kit (NEB Cat.No. E2040S). The donor single strand oligo(ssODN) was synthesized by IDT company(www.idtdna.com). The sequence of the donor ssODN is:

CtcatggatgggctgcctccaggtgacagcaatcagctggcctggtttgatactgacctgGACTACAAGGACCACGACGG
CGATTATAAGGATCACGATATCGACTACAAAGACGACGATGACAAGTGAatcatcctttaggtaagaag
ttttaaaaagccagtttgggtaaaatacttttactctgcc

hESCs were individualized by Accutase treatment, washed once with 1×PBS and spun down at 300 g for 3 minutes, 0.2 million of cells were resuspended in 10ul of R buffer (Invitrogen). cell suspension was added into a 1.5ml Eppendorf tube containing 1µg of gRNA/Cas9 RNP complex and 1µl of 10uM donor SSODN. The mixture of Cas9 protein, gRNA, donor plasmids and cells were then subjected to electroporation using Neon Transfection System (Life technologies) by following the Manufacture's instruction. The survived cells were maintained in mTeSR/Geltrex condition for 10 days. Then, 32 colonies were randomly picked up for PCR screening and DNA sequencing to identify the colony with correct homologous recombination. The positive colonies confirmed by DNA sequencing were further single-cell subcultured and expanded.

3.2.5. Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as in Estaras et. al with some modifications. (Estarás, Benner, & Jones, 2015). 2 x 10^7 cells were double crosslinked with 0.2 mM di (N-succinimidyl) glutarate (DSG, Sigma, 80424) for 45 min followed by 1% formaldehyde (ThermFisher Scientific, 28906) for 15 min. Cell were lysed with cell lysis buffer (10 mM Tris pH

8.0, 10 mM NaCL, 0.2% NP40, protease inhibitors) for ice on 10 min, spun down at 2500 RPM and lysed in nuclei lysis buffer (50 mM Tris 8.1, 2 mM EDTA, 1% SDS, protease inhibitors) for 10 min on ice. Lysate was sonicated in IP dilution buffer (20 mM Tris pH 8.1, 2 mM EDTA, 150 mM NaCal, 1% Triton X-100, 0.1% SDS, protease inhibitors) in a Qsonica Q700 bath sonicator at amplitude 60, 15 seconds ON, 45 seconds OFF for a process time of 3:45, repeated 8 times. Subsequently sonicated lysates were spun down at max RPM for 10 min precleared with magnetic beads (ThermoFisher, 88802) for 1 hour, and subjected to 5 ug α-GFP (Abcam, ab290) overnight. Following overnight incubation with anitbody, a one hour incubation with A/G magnetic beads was performed followed by subsequent washes with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Hcl pH 8.0, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.0, 500 mM NaCl), LiCl wash buffer (0.25M LiCL, 1% NP40, 1% deoxycholate, 1 mM EDTA, 20 mM Tris pH 8.0), and 2x TE washes. DNA was then eluted in fresh elution buffer and uncrosslinked at 65 C for 4 hours with NaCL treatment and treated overnight at 50 C with proteinase K.

3.2.6. RNA-seq analysis

All RNA sequencing was performed at BGI Americas Corporation. Libraries for RNA-Seq were prepared with KAPA Stranded RNA-Seq Kit. The workflow consists of mRNA enrichment, cDNA generation, and end repair to generate blunt ends, A-tailing, adapter ligation, and PCR amplification. Different adapters were used for multiplexing samples in one lane. Sequencing was performed on a BGISEQ-500 for a single end 50 bp run. Reads were filtered to remove reads which contained adapter sequences, high content of unknown bases, and low quality reads. Reads were aligned to the hg19 reference genome using STAR v2.5.2b (Dobin et al., 2013). Differential analysis was performed using edgeR with a threshold of FDR < 0.05 and FC > 2 (Robinson, McCarthy, & Smyth, 2010). Heatmaps were generated using Heatmapper (Babicki et al., 2016). Gene ontology analysis was performed using DAVID (D. W. Huang, Sherman, & Lempicki, 2009).

3.2.7. ChIP-seq analysis

ChIP DNA was sequenced in an Illumina Nextseq500. Reads were aligned to the Human hg19 genome assembly using BWA v0.7.10 (H. Li, 2013). Peak finding, motif finding, and peak annotation were performed using HOMER (Heinz et al., 2010). Binding peaks for β-catenin ChIP-seq were identified using 'findPeaks' command in HOMER with default settings of '-style factor.' Genome browser read density files were created using deepTools (Ramírez et al., 2016) and visualized using IGV (Thorvaldsdóttir, Robinson, & Mesirov, 2013).

Enhancer identification was performed using RFECS based on the use of H3K4me3, H3K27me3, and H3K4me1 (Rajagopal et al., 2013). Identified enhancers were merged if within 1600 bp of each other using bedtools (A. R. Quinlan & Hall, 2010). Identified enhancers were annotated using Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010).

3.3. Results and Discussion

3.3.1. Characterization of in vitro neural differentiation

We utilized our previously developed protocol to generate regionalized hNPCs from hPSCs using BMP inhibition in conjunction with a gradient of WNT signaling established using the WNT agonist CHIR98014 (Cutts et al., 2016; Moya et al., 2014) (Figure 3.1A). We first performed RNA-seq on embryonic stem cell (ES), neuroectodermal cells (NE), anterior- (A), midbrain- (M), and posterior- (P) patterned hNPCs to generate transcriptional signatures to further interrogate. We identified differentially expressed genes (FC > 2 and FDR < 0.05) that transcriptionally defined each cell type (Figure 3.1B). When we examined these transcriptional signatures we observed robust expression of cell type specific genes. ESCs highly expressed the transcription factors NANOG and POU5F1, which are critical components of the core transcriptional regulatory circuit governing pluripotency (Boyer et al., 2005). FOXB2 and PHOXA2 are markers of early neuroectodermal cells and are expressed highly in NE cells (Brunet & Pattyn, 2002; Pohl, Knöchel, Dillinger, & Knöchel, 2002). Well established markers of telencephalon development, FOXG1 and SIX3 are enriched in A- patterned hNPCs (Oliver et al., 1995; W. Tao & Lai, 1992). Genes uniquely upregulated in M- patterned hNPCs included essential genes for mesencephalic and dopaminergic neuron differentiation, LMX1B, EN1, and WNT1 (Chao Guo et al., 2007; Simon, Saueressig, Wurst, Goulding, & O'Leary, 2001; Smidt et al., 2000). Genes uniquely

upregulated in P- patterned hNPCs included important regulators of hindbrain specification and canonical WNT targets including HOXC4, HOXD1, FGF8, LEF1, and SP5 (Galceran et al., 1999; Sunmonu, Li, Guo, & Li, 2011; Tümpel, Wiedemann, & Krumlauf, 2009). We additionally validated expression of several A-, M-, and P- differentially expressed genes identified in transcriptional signatures using qPCR futher confirming that these genes are uniquely upregulated in their respective cell type (Supplemental Figure B.1A). Finally, correlation analysis between biological replicates was high indicating reproducibility of the identified transcriptional signatures of each cell type (Figure 3.1C).

Gene ontology analysis of A-, M-, and P- patterned hNPCs show terms enriched for their respective cell type (Figure 3.1D). The gene signature for anterior patterned hNPCs (A) was enriched for terms related to forebrain anterior/posterior pattern specification and telencephalon regionalization. Anterior/posterior pattern specification and midbrain development terms were enriched in midbrain patterned hNPCs. Finally, canonical WNT signaling and hindbrain development terms were enriched in genes expressed in posterior patterned hNPCs. Taken together these data indicate that the genes identified can confidently be used as transcriptional signatures of each cell type.

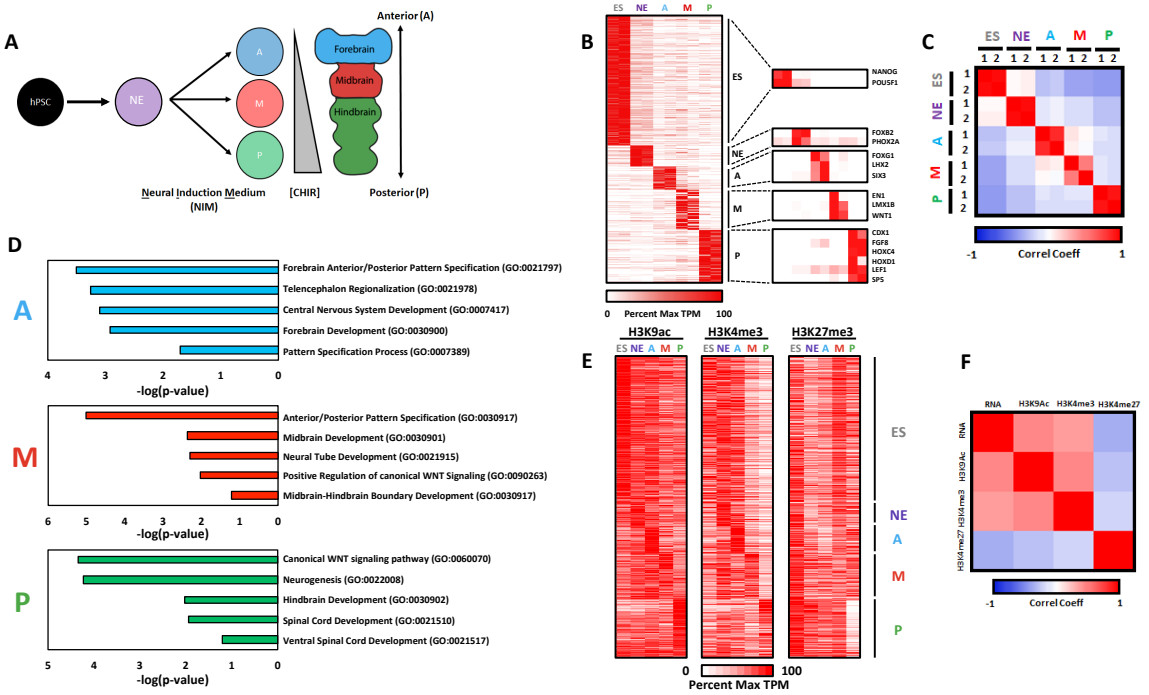

Figure 3.1 Characterization of an in Vitro Model of Human Neural Patterning A) Schematic of differentiation and patterning protocol B) Transcriptional signatures of various cell types identified through differential analysis of RNA-seq C) Correlation analysis of RNA-seq biological replicates D) Gene ontology over enrichment analysis of A-, M-, and P- patterned hNPCs E) ChIP-seq of various cell types of H3K4K9ac, H3K4me3, and H3K27me3 normalized read density in promoters of identified transcriptional signatures F) Correlation analysis of RNA-seq, H3K9ac, H3K4me3, and H3K27me3 ChIP-seq

To further characterize our in vitro model of neural patterning we assessed the epigenetic state of our identified transcriptional signatures by performing chromatin immunoprecipitation sequencing (ChIP-seq) for H3K9ac, H3K4me3, and H3K27me3. H3K9ac is commonly associated with gene activation at gene promoters (Brownell et al., 1996; Jin et al., 2011). Bivalent chromatin domains possessing both the active mark H3k4me3 and repressive mark H3K27me3 are present in ESCs marking developmentally poised genes that are expressed at low levels (Azuara et al., 2006; Bernstein et al., 2005). However generally H3K4me3 is indicative of active chromatin while H3K27me3 marks silent chromatin (Kouzarides, 2007). Consistent with these definitions we found

that around promoters, epigenetic marks H3K9ac and H3K4me3 of transcriptional signature genes are enriched in their respective cell types while H3K27me3 is depleted (Figure 3.1E). Target gene traces for respective cell types ES (NANOG), A- (SIX3), M- (LMX1B), and P- (HOXC cluster) patterned hNPCs additionally demonstrate this finding (Supplemental B.1B). Global correlation between RNA expression and profiled epigenomic marks are in concordance with this, demonstrating positive correlation between RNA and H3K4me3/H3K9ac and negative correlation these with H3K27me3 (Figure 3.1F).

3.3.2. WNT signaling elicits regional transcriptional signatures through β-catenin

To understand how β-catenin levels lead to distinct transcriptional signatures identified we performed neural patterning using incremental amounts of the WNT agonist CHIR98014 followed by RNA-seq. Using the transcriptional signatures previously identified we see activation and deactivation of M- and P- transcriptional signatures at specific thresholds of 0.05 uM – 2 uM and > 3 uM respectively (Figure 3.2A). These findings corroborate previous work that demonstrated that WNT/β-catenin signaling acts a morphogen to activate M- and P- cell identities at specific thresholds of WNT/β-catenin (Kiecker & Niehrs, 2001). Additionally we observe that the default fate of hNPCs in the absence of additional patterning factors is a forebrain identity, supporting the two-step hypothesis proposed by Niewkoop and observed in other hPSC neural differentiation protocols (Nieuwkoop & Nigtevecht, 1954; Y. Tao & Zhang, 2016).

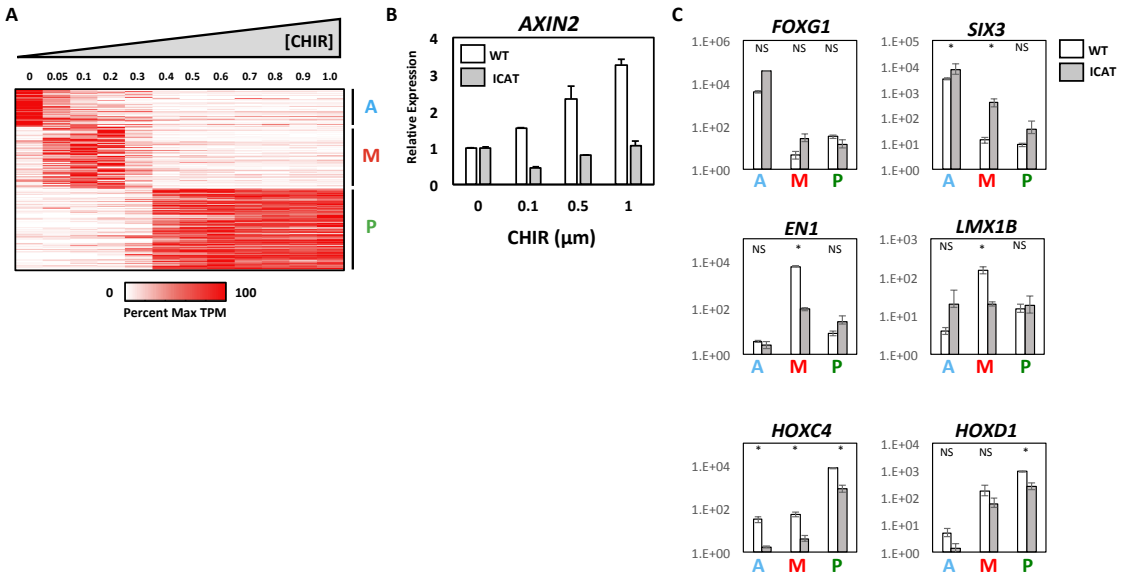

Figure 3.2 WNT Signaling Elicits Regional Transcriptional Signatures through β-catenin A) RNA-seq of identified A-, M-, and P- transcriptional signatures in a gradient of CHIR B) Expression of AXIN2 in ICAT overexpression hESCs (mean ± SEM, n = 3 independent experiments). C) Expression of anterior, midbrain, and posterior markers of the neural tube in WT and ICAT overexpression hESCs. Populations were compared with neurons differentiated from untreated (N) NPCs using Student's t test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

To understand how β-catenin was able to elicit these different transcriptional states we aimed to interrupt its ability to affect transcription. β-catenin functions both in adherens junctions and as a signaling component of WNT signaling and activates expression of WNT target genes through binding additional transcription factors, most commonly TCF/LEF. It is difficult to separate its two functions in cellular processes (Tomas Valenta et al., 2012). To overcome this we decided to constitutively overexpress the protein inhibitor of β-catenin and TCF (ICAT) which is known to bind to the C-terminus of β-catenin and inhibit its ability to interact with transcription factors bound to chromatin (Daniels & Weis, 2002; Tago et al., 2000). Thus interfering with β-catenin's transcriptional activity while leaving its ability to bind adherens junctions uninterrupted (Supplemental Figure B.2A). We observed stable overexpression of ICAT by qPCR following transduction (Supplemental Figure B.2B). Additionally, H1 ICAT overexpression hPSCs show impaired expression of the WNT target gene AXIN2 following treatment with CHIR98014,

demonstrating that β-catenin's ability to influence expression of WNT target genes is reduced in the presence of ICAT (Figure 3.2B).

We used our characterized model of neural patterning and differentiated H1 WT and H1 ICAT hPSCs to A-, M-, and P- patterned hNPCs to delineate the role of WNT/β-catenin mediated transcription in cell fate decisions by performing qPCR on target genes identified in our unique transcriptional signatures. A- patterned hNPCs most highly expressed telencephalic markers FOXG1 and SIX3 (Fig 3.2C). Overexpression of ICAT did not affect FOXG1 expression in A-patterned hNPCs while SIX3 was expressed more highly in ICAT with ICAT overexpression. This is consistent with previous studies, which have indicated the necessity of WNT inhibition in development of the telencephalon (Glinka et al., 1997; Mukhopadhyay et al., 2001). Markers of the midbrain EN1 and LMX1B are most highly expressed in M- patterned hNPCs, and are significantly downregulated by ICAT overexpression. Our data support previous findings that β-catenin directly regulates the expression of EN1 (Alves dos Santos & Smidt, 2011). Additionally LMX1B is a known regulator of acquisition of midbrain dopaminergic neurons, ICAT overexpression inhibits its' expression consistent with the idea that β-catenin plays important roles in specifying midbrain dopaminergic neuron fate (Joksimovic & Awatramani, 2014; C. H. Yan, Levesque, Claxton, Johnson, & Ang, 2011). Finally, HOX cluster genes HOXC4 and HOXD1, previously identified in our P- transcriptional signature are most highly expressed in P-patterned hNPCs in H1 WT hPSCs, while expression is downregulated in ICAT overexpression hPSCs. (Figure 3.2C) HOXC4 and HOXD1have previously been shown to be activated in response to WNT/β-catenin signaling, these results indicate that by inhibiting β-catenins' transcriptional activity the expression of these signature genes is also inhibited (Janssens, Denayer, Deroo, Van Roy, & Vleminckx, 2010; Otero, Fu, Kan, Cuadra, & Kessler, 2004).

β-catenin activates expression of M- and P- target genes, as evidenced by this data. By interfering with β-catenin's ability to activate transcription through ICAT overexpression we inhibit acquisition of M- and P- fates at respective levels of WNT/β-catenin signaling. This suggests that β-catenin acts differently at different levels of WNT signaling, to activate specific transcriptional signatures.

3.3.3. CTNNB1 binding genome wide

To understand how β-catenin acts to activate expression of M- and P- cell fates during neural patterning we performed ChIP-seq in A-, M-, and P- patterned hNPCs. We initially had difficulty optimizing a ChIP protocol to capture β-catenin binding events during neural patterning, potentially due to its indirect binding of chromatin through transcription factors additional transcription factors (Cadigan & Waterman, 2012). To overcome this technical challenge we added a 3XFLAG sequence to the C- terminus of β-catenin using a CRISPR mediated targeting, we refer to this new hPSC line as CTNNB1-3XFLAG (Supplemental Figure B.3A). We performed extensive characterization to ensure proper editing and maintenance of pluripotency by sequencing in a selected clone. Sequencing results confirmed successful incorporation of the 3XFLAG at the C- terminus of β-catenin, and α-FLAG staining shows localization to the cytoplasm and nucleus, in line with β-catenins' known role in adherens junctions and as a transcriptional effector of WNT/b-catenin signaling (Supplemental Figure B.3B-B.3C). Cells retained a normal karyotype and morphology characteristic of hPSCs, with compact colonies and distinct edges (Supplemental Figure B.3D-B.3E). Flow cytometry for TRA1-81 and immunofluorescence for NANOG, OCT4, and SOX2 indicate maintenance of pluripotency. Finally CTNNB1-3XFLAG cells were subjected to an undirected differentiation. Differentiated cells stained positive for SMA, AFP, and B3T indicating that these cells retain the ability to differentiate to the 3 germ layers, further demonstrating that these cells retained characteristic pluripotency. The CTNNB1-3XFLAG hPSC line serves as a valuable tool to directly map β-catenins' binding during neural patterning, and will also aid the field in future investigations into the transcriptional activity of β-catenin in different developmental contexts.

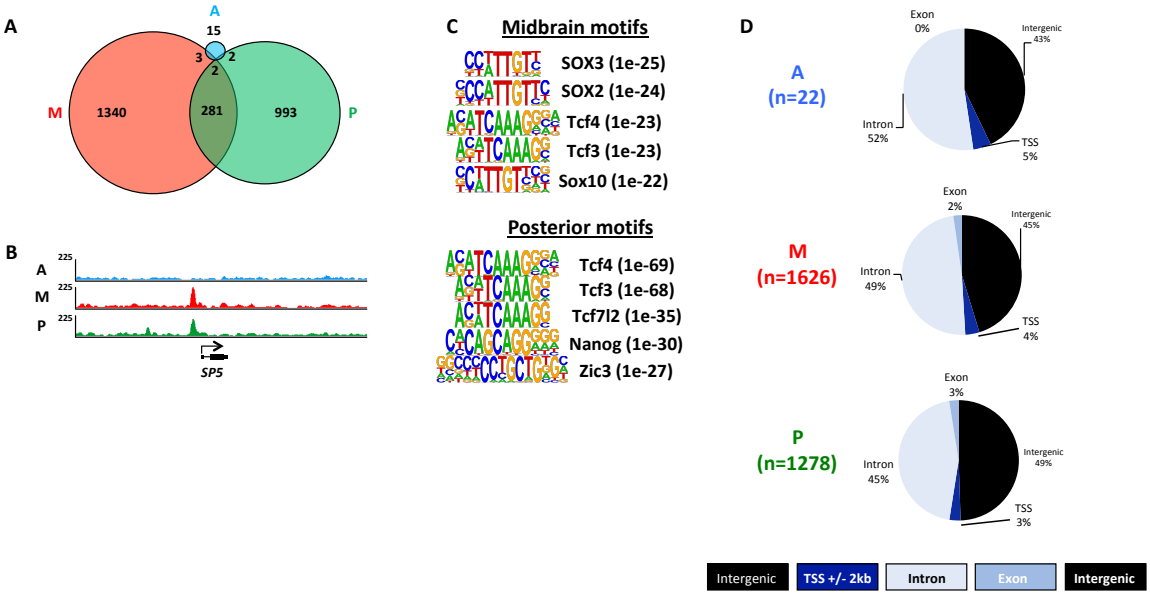

Figure 3.3 Characterization of β-catenin binding genome wide during human neural patterning A) Triple venn diagram of Identified β-catenin peaks in A-, M-, and P- patterned hNPCs B) Genome browser capture showing β-catenin binding in the promoter of SP5 C) Motif analysis of β-catenin peaks identified in M- and P- patterned hNPCs D) Annotation of β-catenin peaks identified in A-, M-, and P- patterned hNPCs

CTNNB1-3XFLAG hPSCs were treated with CHIR98014 for 48 hours and subjected to ChIP followed by qPCR using primers against the promoter of the known WNT target gene *SP5*. As expected CHIR treated cells express WNT targets genes *AXIN2* and *SP5* (Supplemental Figure B.4A-B.4B). Additionally CHIR treated cells show significantly higher binding of β-catenin in the promoter of SP5 than non-treated cells (Supplemental Figure B.4C). We then differentiated this cell line as previously described to A-/M-/P- regional identities and performed α-FLAG ChIP-seq to identify β-catenin binding genome wide and characterize its activity in A/P neural patterning. Global analysis revealed very few peaks in anterior patterned hNPCs (n=22) while identifying a much greater number of peaks in midbrain (n=1621) and posterior (n=1274) patterned cells (Figure 3.3A). We identified a strong β-catenin peak in the promoter of the canonical WNT target gene SP5, and confirmed strong b-catenin binding there by ChIP-qPCR (Figure 3.3B and Supplemental Figure B.4D). Interestingly we identified 281 overlapping peaks between M- and P- patterned hNPCs and negligible overlap with A- patterned hNPCs, suggesting

that the concentration of β-catenin in the nucleus results in unique binding events. To investigate this differential binding activity we performed motif analysis on β-catenin peaks identified in M- and P- patterned hNPCs. Top motifs identified in β-catenin peaks from M- patterned hNPCs include SOX and TCF motifs. SOX transcription factors are closely related to TCF, both being in the HMG family (Kormish, Sinner, & Zorn, 2010) SOX transcription factors have been demonstrated to act to regulate β-catenin mediated transcription by modulating the WNT signaling pathway, inhibiting the ability of β-catenin to elicit transcription in the TOP:flash reporter (Zorn et al., 1999) Although in some instances SOX transcription factors, such as SOX2 are hypothesized to activate expression of genes such as cyclinD1 (Iguchi et al., 2007; Kormish et al., 2010). Top motifs identified in β-catenin peaks from P- patterned hNPCs include the TCF motif followed by Nanog and Zic3 (Figure 3.3C). β-catenin binding is most well characterized to TCF/LEF transcription factors (Cadigan & Waterman, 2012; Schuijers et al., 2014) It is interesting that a Zic3 motif is enriched as Zic3 is suggested to have a role in modulating WNT activity, and indeed, Zic3 knockdown results in reduction of head size in Xenopus, similar to interruption of WNT antagonism in the specification of anterior neural tube (Fujimi, Hatayama, & Aruga, 2012). Differential enrichment of motifs suggests a possible mechanism for differential peak binding at different concentrations of WNT/β-catenin signaling. Annotation of identified β-catenin peaks revealed that only a minority of β-catenin peaks identified in M- and P- patterned hNPCs were located in promoters (4 % and 3 % respectively) while the majority of peaks were in intronic and intergenic regions (Figure 3.3D).

Genes annotated to β-catenin peaks identified in M- patterned hNPCs were enriched for WNT signaling pathway, anterior/posterior patterning specification, neural tube formation, and dopaminergic neuron differentiation (Supplemental Figure B.4C). While genes annotated to β-catenin peaks identified in P- patterned hNPCs were enriched for canonical WNT signaling, anterior posterior patterning, and hindbrain development (Supplemental Figure B.4C). Gene ontology analysis of genes annotated to β-catenin peaks reveals differential enrichment of terms associated with A/P neural patterning in M- and P- patterned hNPCs.

3.3.4. Identification of enhancers in neural patterning

Since a majority of identified β-catenin peaks were annotated to intronic or intergenic regions we hypothesized that β-catenin was interacting with enhancers to influence cell fate decisions. Indeed, previous work has indicated that β-catenin interacts with enhancers to promoter enhancer-promoter looping of WNT target genes (Estarás et al., 2015; Yochum, 2011; Yochum et al., 2010).

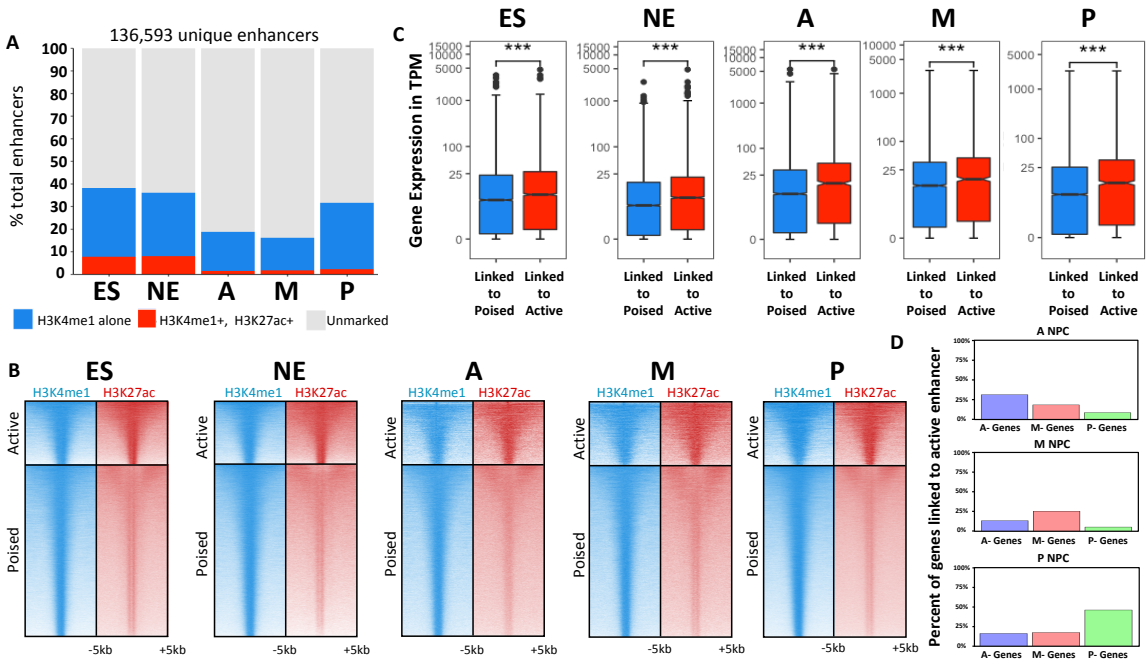

Figure 3.4 Identification and Characterization of Enhancers during Human Neural Patterning A) Total number of putative enhancers identified during neural progenitor differentiation and patterning categorized by H3K27ac and H3K4me1 deposition B) Density of ChIP-seq reads for H3K4me1 and H3K27ac relative to the midpoint of putative poised and active enhancers C) Box plots of mRNA expression, measured in TPM at linked genes of putative poised and active enhancers *** p-value < 2.2e-16 wilcoxon rank sum test. D) Percent of transcriptional signature genes marked by an active enhancer ES, embryonic stem cells; NE, neuroectoderm; A, anterior hNPCs; M, midbrain hNPCs; P, posterior hNPCs.

Enhancers are widespread distal regulatory elements that control the spatiotemporal expression of genes in development and disease (Kleinjan & van Heyningen, 2005; Sakabe, Savic, & Nobrega, 2012; Shlyueva, Stampfel, & Stark, 2014). These genomic loci are bound by

tissue specific transcription factors, which subsequently recruit cofactors such as p300 to activate gene expression (Merika, Williams, Chen, Collins, & Thanos, 1998). Enhancer identification has historically been challenging due to the their long range interactions across the non-coding regions of the genome and no known sequence specific code delineating their activity in different developmental contexts (Pennacchio, Bickmore, Dean, Nobrega, & Bejerano, 2013) However observations that combinatorial presence or absence of histone post translational modifications (PTMs) in defined genomic loci mark enhancers, in conjunction with ChIP-seq to map these modifications can be used to identify enhancers (Creyghton et al., 2010a; Heintzman et al., 2009, 2007). We profiled H34me1, H3K27me3, and H3K27ac histone PTMs in our system of human A/P patterning using ChIP-seq and identified enhancers using a random forest based algorithm for enhancer identification from chromatin state (RFECS) (Rajagopal et al., 2013). Using this machine learning based approach to enhancer identification we identified 136,593 unique enhancers (Figure 3.4A). This is consistent with the notion that the genome contains hundreds of thousands of enhancers that enable precise control of gene expression in different developmental contexts (ENCODE Project Consortium, 2012; Shen et al., 2012; Zhu et al., 2013). Previous work has demonstrated that the presence or absence of H3K27ac marks active vs. poised enhancers (Creyghton et al., 2010b). To that end we classified enhancers as active or poised and found, similar to previous work, that most enhancers are in each cell type are poised while only a subset are marked as active (Figure 3.4B) (A. Wang et al., 2015). Additionally genes linked to putative active vs. poised enhancers are significantly more highly expressed (Figure 4C). Further analysis of genes linked to active enhancer via gene ontology revealed that genes linked to active enhancers are enriched for regional specific terms (Supplemental Figure B.5A). Motif analysis of active enhancers showed that motifs in A-, M-, and P- patterned hNPCs were enriched for SOX motifs, consistent with their known role as regulators of neural enhancers (Wegner, 2011). We further examined the distribution of active enhancers within our previously identified transcriptional signatures and found that genes linked to active enhancers were enriched with respect to cell state (Figure 3.4D). These results functionally define active and poised enhancers in human A/P patterning and serve well to further interrogate the interaction between β-catenin

and enhancers in cell fate decisions. Furthermore this analysis serves as a valuable resource to investigate the mechanisms of cell competence during regionalization in neural development.

3.3.5. β-catenin binding in enhancers falls into multiple classes

As we hypothesized, the vast majority of β-catenin peaks previously annotated to intragenic and intronic regions were re-annotated to identified enhancers (M- 95%, P- 94%) (Figure 3.5A). These data support a divergence from the traditional idea of β-catenin controlled gene expression where b-catenin binds promoters to activate gene expression (Cadigan, 2012; Tomas Valenta et al., 2012). A new model posits that β-catenin instead binds enhancers to act as a primer, in concert with other transcription factors, to control expression of target genes (Nakamura et al., 2016; Nakamura & Hoppler, 2017; Ramakrishnan & Cadigan, 2017).

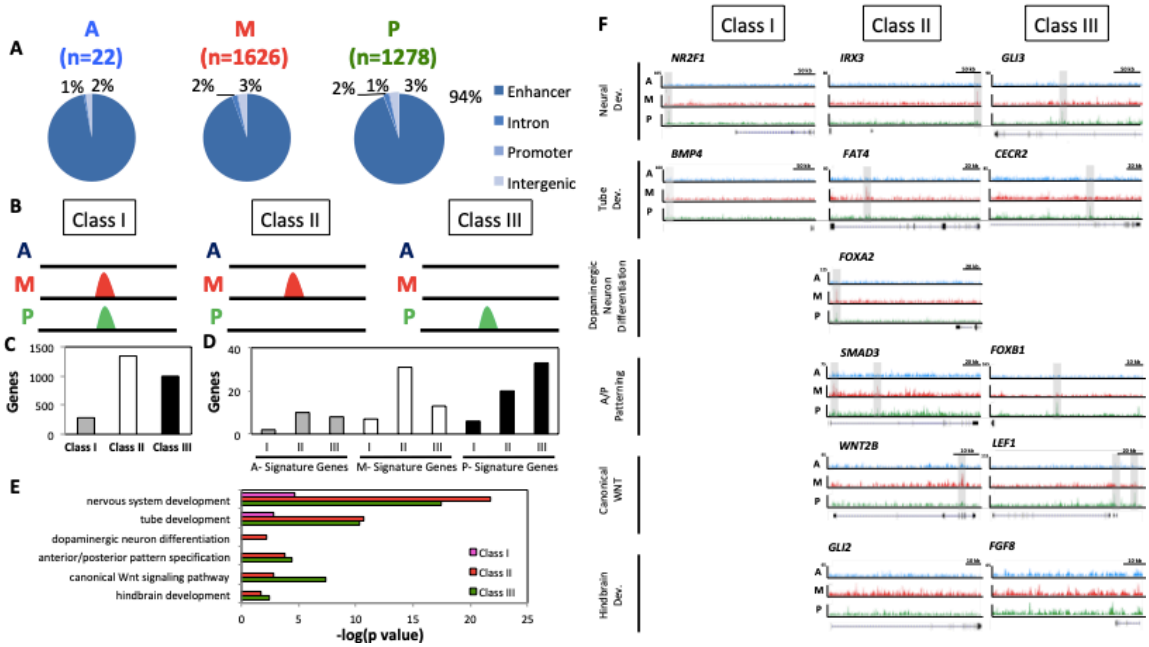

Figure 3. 5 Identification and characterization of β-catenin peak classes A) Reannotated β-catenin ChIP-seq peaks following identification of enhancers B) Model of β-catenin binding in a graded manner C) Global classes of β-catenin peaks D) β-catenin in identified transcriptional signatures E) Gene ontology over enrichment analysis of genes bound by different classes of β-catenin peaks F) Representative genome browser captures of class specific genes in gene ontology analysis

β-catenin peaks were observed to mostly be unique to M- and P- patterned hNPC states, but with significant overlap of peaks between the two conditions. We decided to further investigate this phenomenon by classifying β-catenin peaks into classes based on overlap or uniqueness between M- and P- patterned hNPCs. We defined class I peaks as having an overlapping β-catenin peak in both the M- state and P- state. While class II and class III have a β-catenin peak in only the M- state or P- state respectively. (Figure 5B) We identified 283 class I, 1346 class II, and 997 class III β-catenin peaks. (Figure 3.5C) When we examined the distribution of peaks by class in previously identified transcriptional signatures we observed that the M-cluster is enriched for class II β-catenin regulated genes (31/126) while the P- cluster is enriched for class III β-catenin regulated genes (33/164) (Figure 5D). GO analysis of peaks by class indicate that all classes are enriched for general terms related to neural and tube development (Figure 3.5E). While class II and III specific affect regionalization of the neural tube and acquisition of specific regional identities such as dopaminergic neuron differentiation enriched in class II and canonical WNT signaling pathway and hindbrain development were enriched in class III. This suggests that β-catenin regulates neural and tube development generally with class I peaks while class II and III peaks regulate genes involved in proper patterning of the developing neural tube (Figure 3.5F)

3.4. Conclusion

WNT signaling produces unique transcriptional outputs at graded levels of WNT signaling. The traditional view of canonical WNT target gene activation fails to capture the observed complexity of graded WNT signaling. Several examples of gradients of WNT signaling during development demonstrate the necessity of graded activation of WNT targets. Mutations of varying severity in APC led to different levels of β-catenin in mouse models of hematopoietic stem cell (HSC) development. Luis et. al. demonstrated that different levels of WNT/β-catenin signaling led to different HSC function, with low levels of WNT leading to enhanced HSC function, mid levels leading to enhanced t-cell differentiation, and high levels impairing HSC function (Tiago C. Luis et al., 2011) Additionally it is well known that graded levels of WNT signaling regionalize the developing neural tube (Moya et al., 2014; Nordström et al., 2002). Here we used an in vitro

model of neural patterning taking advantage of the distinct transcriptional outputs at different levels of WNT signaling and profiled the binding of β-catenin to understand how it acts to regulate cell fate in a graded manner.

Studies to date have examined β-catenin binding in the presence or absence of WNT activation, which does not take into the account the known gradient transcriptional activity of WNT/β-catenin signaling, observed in multiple developmental contexts. B-catenin chip-seq has been performed in colorectal cancer cell lines (Bottomly et al., 2010; Schuijers et al., 2014), HEK 293T (Doumpas et al., 2019; Schuijers et al., 2014), and murine intestinal crypt cells (Schuijers et al., 2014). Additionally β-catenin binding has been examined in multiple hPSC derivatives, including primitive streak formation (Funa et al., 2015) and mesendodermal induction (Estarás et al., 2015). Finally several β-catenin ChIP-seq studies have been performed in xenopus gastrulation (Kjolby & Harland, 2017; Nakamura et al., 2016) and xenopus endoderm development (Stevens et al., 2017). Clearly β-catenin acts across multiple developmental contexts, consistent with the ability of WNT/β-catenin signaling ability to regulate multiple cell and developmental processes.

In line with previous results we observe that β-catenin binds to enhancers to prime their activity (Nakamura et al., 2016). This mechanism may explain how β-catenin is able to serve as a transcriptional regulator in a vast number of developmental contexts observed including hematopoiesis (Tiago C. Luis et al., 2011), gastrulation (Funa et al., 2015; Nakamura et al., 2016), and mesendodermal induction (Estarás et al., 2015). Interestingly b-catenin binding events are mostly unique in M- and P- patterned hNPCs due entirely to only changes in concentration of WNT/b-catenin signaling provided during neural patterning (Figure 3A). Motif analysis suggests that differential motif enrichment between M- and P- patterned hNPCs could be due to interactions with different transcription factors. At lower concentrations of WNT signaling M-hNPCs are specified and SOX motifs are most enriched while at higher concentrations P-patterned hNPCs are enriched for TCF/LEF motifs (Figure 3B). SOX motifs have previously been shown to act in concert with β-catenin, though their activity in as transcriptional activators has not been well characterized (Kormish et al., 2010) We also find that SOX motifs are enriched in active

enhancers that we identified (Supplemental Figure 5B) and M- β-catenin peaks are enriched for SOX motifs, this suggests interaction between these identified enhancers and identified β-catenin peaks. A higher threshold of b-catenin binding could be required for binding to TCF/LEF transcription factors that are enriched in b-catenin peaks identified in P- patterned hNPCs. An open question remains as to how β-catenin becomes removed from these lower threshold class II binding events to turn off genes at this level of WNT/β-catenin signaling.

Here we characterize β-catenin binding in a graded fashion using our previously characterized in vitro model of neural development. Specific levels of WNT activation lead to differential cell fate. We find that in line with previous observations, most β-catenin binding lies within enhancers to influence cell fate. Additionally we observe β-catenin binding falls into different classes depending on the presence or absence of peaks at different levels of WNT signaling. A minority of peaks overlap between graded levels of WNT activation while most are unique to patterned hNPC populations, corresponding to different levels of WNT signaling. This study expands the traditional binary view of canonical WNT signaling and helps illuminate WNT/β-catenin activity in other developmental and disease contexts.

CHAPTER 4

THE ROLE OF THE WNT/BCATENIN INDUCED TRANSCRIPTION FACTOR, SP5 IN NEURAL PATTERNING

4.1. Introduction

Signaling pathways convey complex information resulting in the dissemination of information required during development for the generation of a properly formed adult organism. The WNT/ β-catenin signaling is one such pathway, conserved across all metazoans which transmits such information. It plays roles in a multitude of cell and developmental processes including formation of the primitive streak, gastrulation, and patterning of anterior/posterior and dorsal/ventral axes(Loh, van Amerongen, et al., 2016). Mutations in this pathway compromising parts of the pathway can lead to severe congenital defects. While mutations resulting in aberrant activation of different components in this pathway can result in the developmental of multiple forms of cancer (Clevers & Nusse, 2012; Wiese, Nusse, & van Amerongen, 2018).

Activation of the WNT/ β-catenin signaling pathway results in accumulation of β-catenin in the cytoplasm of cells, which subsequently translocates to the nucleus, where it interacts with TCF/LEF transcription factors to activate WNT target genes. The activation of WNT target genes by β-catenin is well known to play critical developmental roles, for example in mesoendoermal differentiation (Estarás et al., 2015). Although many studies have focused on characterizing transcriptional activation by β-catenin, less work has focused on how these signals are turned off in an appropriate temporal fashion.

The SP1 like transcription factor, SP5, is activated following WNT stimulation and acts as a selective transcriptional repressor (Dunty, Kennedy, Chalamalasetty, Campbell, & Yamaguchi, 2014b; Fujimura et al., 2007). SP5 is expressed in multiple tissues throughout mouse development localized to regions where WNT signaling is active (C. J. Thorpe, Weidinger, & Moon, 2005; Weidinger, Thorpe, Wuennenberg-Stapleton, Ngai, & Moon, 2005a). Recent work in human pluripotent stem cells demonstrates that SP5 acts as a WNT induced negative regulator of WNT signaling, reining in expression of previously activated WNT target genes (Huggins et al., 2017). Work in mouse embryos posits an alternate hypothesis, that SP5 plays a role as a co-

activator, fine tuning WNT signaling by interacting with enhancers to help activate WNT target genes (Kennedy et al., 2016). These contradictory roles suggest it is possible that SP5 has different functions in different mammalian or developmental contexts.

Here we examine the role of SP5 in early human neural patterning. Previous work has demonstrated that a gradient of WNT signaling specifies regional identity in the developing neural tube, through β-catenin binding. We demonstrate the SP5 is strongly induced by WNT signaling in A/P patterning and is directly regulated by β-catenin binding. Preliminary studies indicate that genetic knockout of SP5 results in impaired neural patterning, indicating its essential role in neural development is in activating regionally specific genes. This work further demonstrates SP5 as an essential regulator of canonical WNT signaling; however more in depth characterization is required to understand its complete mechanism of action.

4.2. Experimental Methods

4.2.1. Cells and culture conditions

hPSCs were passaged in feeder free conditions in Essential 8 medium (Life Technologies) on Matrigel (BD Biosciences). Cells were routinely passaged with Accutase (Millipore) and replated at a density of 4.25 x $10^4/cm^2$ every 3-4 days.

4.2.2. Neural progenitor cell (NPC) generation, expansion, and differentiation

Neural differentiation and patterning were carried out as described previously (Cutts et al., 2016). Briefly, to initiate neural differentiation, hPSCs were cultured on Matrigel (BD Biosciences) in Essential 8 (Life Technologies). Cells were then detached with treatment with Accutase (Millipore) for 5 min and resuspended in E8 and replated at 2 x 10^6 cells per well of a 6 well ultra low attachment plate (Grenier). The plates were then placed on an orbital shaker set at 95 rpm in a 37°C/5% CO^2 tissue culture incubator A fresh half media change was performed after 24, and media was exchanged to neural induction media (1% N2/1% B27 without vitamin A/DMEM:F12) supplemented with 5 μM Y-267632 (Stemgent), 50 ng/ml recombinant mouse Noggin (R&D Systems), 0.5 μM Dorsomorphin (Tocris Bioscience)] after 48 hours. Half media changes were subsequently performed every day. After 2 days in suspension in neural induction media, the EBs

were patterned by the addition of IWP2 (Sigma) or CHIR98014 (Sigma). Cultures were maintained for an additional 5 days before further characterization or analysis.

4.2.3. Quantitative PCR (qPCR)

RNA was isolated from cells using Nucelospin RNA® (Macherey-Nagel. Reverse transcription was performed with iScript™ cDNA Supermix (BioRad). Quantitative PCR was carried out using SYBR probes (IDT) a CFX384 Touch™ (BioRad) and iTaq Universal SYBR Green Supermix (BioRad), with a 2 min gradient to 95°C followed by 40 cycles at 95°C for 5s and 60°C for 30s. Gene expression was normalized to 18S rRNA levels. Delta Ct values were calculated as $Ct^{target} - Ct^{18s}$. All experiments were performed with two technical replicates. Relative fold changes in gene expression were calculated using the 2-ΔΔCt method. Data are presented as the average of the biological replicates ± standard error of the mean (S.E.M).

4.2.4. Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as in Estaras et. al with some modifications. (Estarás, Benner, & Jones, 2015). 2 x 10^7 cells were double crosslinked with 0.2 mM di (N-succinimidyl) glutarate (DSG, Sigma, 80424) for 45 min followed by 1% formaldehyde (ThermFisher Scientific, 28906) for 15 min. Cell were lysed with cell lysis buffer (10 mM Tris pH 8.0, 10 mM NaCL, 0.2% NP40, protease inhibitors) for ice on 10 min, spun down at 2500 RPM and lysed in nuclei lysis buffer (50 mM Tris 8.1, 2 mM EDTA, 1% SDS, protease inhibitors) for 10 min on ice. Lysate was sonicated in IP dilution buffer (20 mM Tris pH 8.1, 2 mM EDTA, 150 mM NaCal, 1% Triton X-100, 0.1% SDS, protease inhibitors) in a Qsonica Q700 bath sonicator at amplitude 60, 15 seconds ON, 45 seconds OFF for a process time of 3:45, repeated 8 times. Subsequently sonicated lysates were spun down at max RPM for 10 min precleared with magnetic beads (ThermoFisher, 88802) for 1 hour, and subjected to 5 ug α-GFP (Abcam, ab290) overnight. Following overnight incubation with anitbody, a one hour incubation with A/G magnetic beads was performed followed by subsequent washes with low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Hcl pH 8.0, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.0, 500 mM NaCl), LiCl wash buffer (0.25M LiCL, 1% NP40, 1% deoxycholate, 1 mM EDTA, 20 mM Tris pH 8.0), and 2x TE washes. DNA

was then eluted in fresh elution buffer and uncrosslinked at 65 C for 4 hours with NaCL treatment and treated overnight at 50 C with proteinase K.

4.3. Results and Discussion

4.3.1. SP5 is strongly induced in neural patterning

Using our previously developed in vitro model of A/P neural patterning, and assaying for SP5 gene expression indicates that SP5 is strongly induced in response to WNT stimulation and most highly expressed in midbrain and posterior patterned neural cells (Figure 4.1A). These findings corroborate studies that demonstrate strong induction of SP5 upon WNT stimulation (Dunty, Kennedy, Chalamalasetty, Campbell, & Yamaguchi, 2014a; C. J. Thorpe et al., 2005). β-catenin ChIP-seq in patterned hNPCs demonstrates that SP5 a strongly bound target of canonical WNT signaling in midbrain and posterior patterned hNPC fates (Figure 4.1B). This provides direct transcriptional evidence of WNT/β-catenin regulated expression of SP5. Furthermore, an AAV- based targeting strategy was used by Huggins et. al. to generate a SP5-YFP fusion protein (Huggins et al., 2017). We used this engineered line to assay SP5 levels with flow cytometry, which confirms gene expression results demonstrating strong induction of SP5 in midbrain and posterior patterned hNPCs (Figure 4.1C).

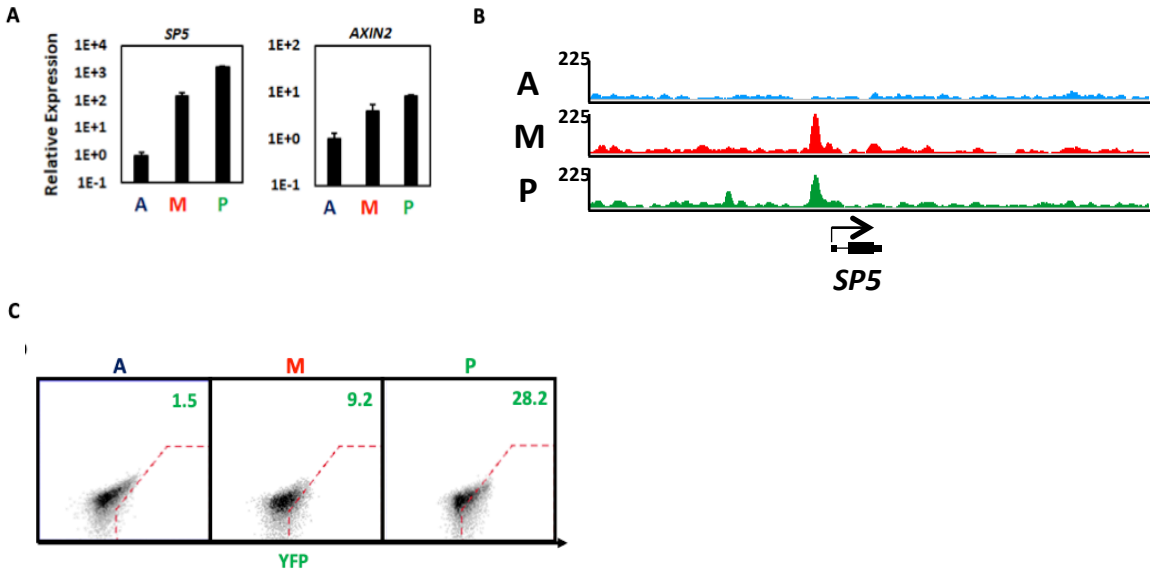

Figure 4.1 Characterization of the Role of the Negative Regulator of WNT Signaling SP5 in Human Neural Patterning A) Expression of WNT targets AXIN2 and SP5 in H1 WT hPSCs differentiated to A-, M-, and P- identities (mean ± SEM, n = 3 independent experiments) B) IGV trace of SP5 locus in β-catenin ChIP-seq performed in A-, M-, and P- patterned hNPCs C) Flow cytometry of A/P patterned hNPCs generated from SP5-YFP fusion hPSCs

To investigate the role of SP5 in neural patterning we used a line generated by Huggins et al. which used CRISPR/Cas9 to engineer cells carrying a loss of function SP5 by truncating the zinc finger (ZF) binding domain in the *SP5* gene (Figure 4.2A) (Huggins et al., 2017). Repair by non-homologous end joining yielded a *SP5* allele that expressed a truncated SP5 protein lacking the ZF binding domain, designated SP5 DZF, functionally equivalent to a knockout of this protein. Sequencing of PCR products of the genomic region containing the targeted region confirmed that several clones contained deletions in both alleles of the Zn finger-encoding region (data not shown). To investigate the degree to which SP5 acts downstream of WNT signaling to specify posterior identities of hPSC-derived cell, we differentiated the SP5 DZF hESC line to neural cells of various A/P neural cell identities and performed qPCR for known targets of A/P patterning. In midbrain patterned hNPCs SP5 dzf impairs expression of known midbrain markers EN1 and LMX1B (Figure 4.2 B). Similarly in posterior patterned hNPCs induction of markers of hindbrain

and spinal cord HOXA2 and HOXC4 are impaired in SP5 DZF hESCs (Figure 4.2 B). Comparison of gene expression using qPCR thus revealed impairment of M- and P- hNPC cell identity in SP5 dzf cell line in patterned NPCs, suggesting that SP5 plays a role inactivating expression of these genes in neural patterning.

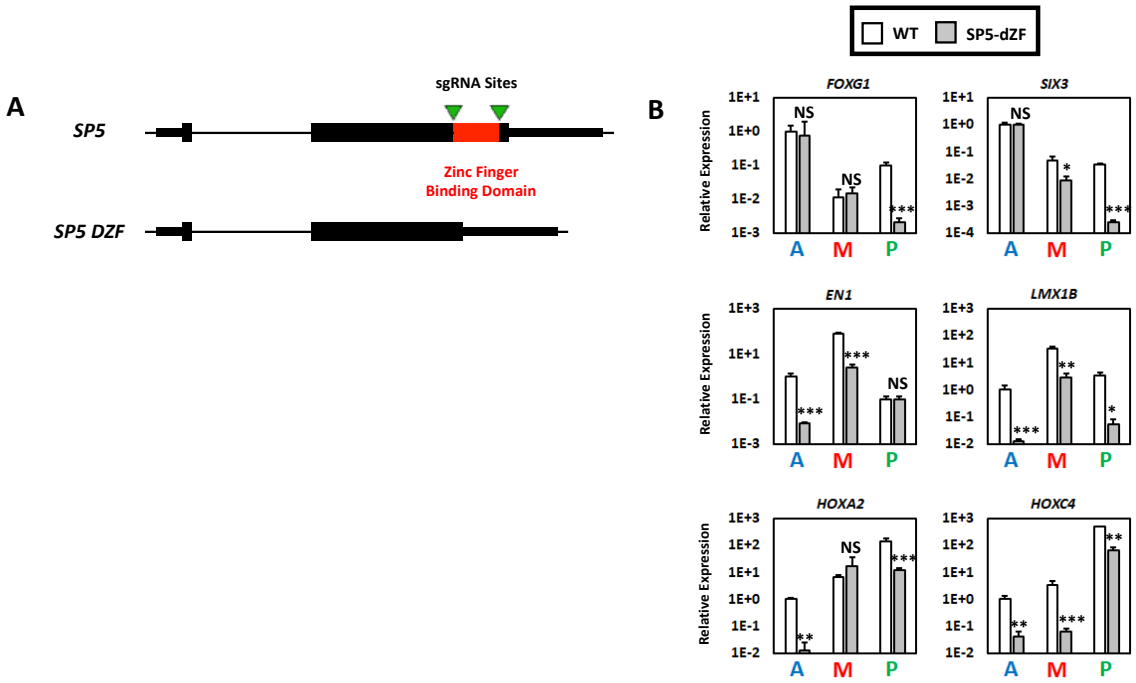

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Figure 4.2 SP5 DZF impairs Neural Patterning A) CRISPR based targeting strategy to generate truncated SP5 DZF hESC line B) Expression of cortical-, forebrain-, midbrain-, hindbrain-related neuronal genes in hNPCs differentiated to A-, M-, and P- cultures derived from H1 WT and H1 SP5-dZF hPSCs (mean ± SEM, n = 3 independent experiments). Populations were compared with H1 WT hPSCs using Student's t test. ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001.

4.3.2. SP5 ChIP-qPCR in hES and patterned hNPCS

To assess SP5 binding in neural patterned cells we treated SP5-YFP hESCs for 48 hours with CHIR98014 and performed ChIP. Cells were isolated and DNA was precipitated using an α-GFP ChIP quality antibody. ChIP-qPCR was performed against the promoter of SP5. WNT activation with CHIR98014 had higher amounts of SP5 bound to the promoter of SP5 than

untreated cells (Figure 4.3A). This is consistent with the notion that SP5 regulates its own expression. We proceeded to examine if SP5 binding is conserved in patterned hNPCs. Using our established procotol we patterned hNPCs using a gradient of WNT in the SP5-YFP fusion hESC line, CHIP followed by qPCR for the promoter of SP5 indicates that SP5 pulldown in the promoter of SP5 in midbrain and posterior patterned hNPCs is higher than anterior patterned hNPCs (Figure 4.3B). The SP5-YFP fusion line and protocols developed can thus be used in future ChIP-seq studies to enable complete understanding of the mechanistic role of SP5 in human A/P patterning.

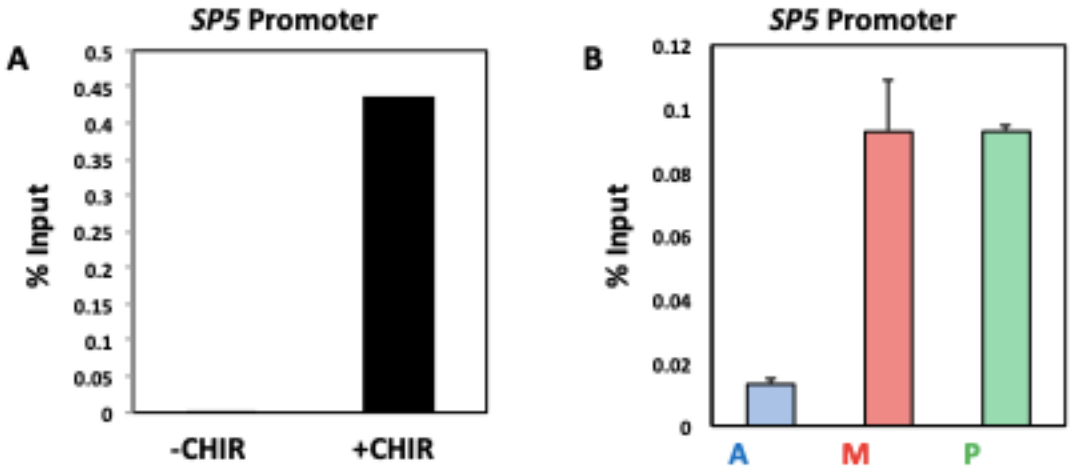

Figure 4.3 Genomic binding analysis of SP5 in human neural patterning A) anti-GFP ChIP-qPCR on the promoter of SP5 in SP5-YFP fusion hPSCs treated with or without 1 uM CHIR for 48 hours (mean ± SEM, n = 3 independent experiments) B) anti-GFP ChIP-qPCR on the promoter of SP5 in A/P patterned hNPCs generated from SP5-YFP fusion hPSCs (mean ± SEM, n = 3 independent experiments)

4.4. Conclusion

5.

WNT/ β-catenin signaling is required for proper development and maintenance of many adult organs; here we examine the role of the WNT induced transcription factor SP5 in the events of early human neural patterning. WNT/ β-catenin activation has been demonstrated to be

essential to the patterning of the early human neural tube (D. Brafman & Willert, 2017; Mulligan & Cheyette, 2012). It has been demonstrated to essential for the proper induction of midbrain, as illustrated by the complete loss of metencephalic regions following loss of WNT1 expression (A P McMahon et al., 1992). Additionally WNT3a secreted from the paraxial mesoderm stimulates expression of Meis3, which in turn is essential for proper formation of the hindbrain (Elkouby et al., 2010). WNT/ β-catenin is thus critical for the proper activation of transcriptional programs related to neural patterning. How these gene programs are appropriately turned off subsequent to WNT stimulated activation remained an open question.

Several mechanisms of negative feedback in WNT/ β-catenin signaling have been identified that target WNT ligands (SFRPs, DKK, NOTUM), FZD receptors (RNF43, APCDD1), and parts of the intracellular signaling cascade (AXIN2) (Huggins et al., 2017; Lustig et al., 2002). However these mechanisms do not address the deactivation of target genes already turned on by WNT signaling. SP5 is a WNT induced transcriptional negative regulator of WNT target genes in early human development (Huggins et al., 2017; Weidinger, Thorpe, Wuennenberg-Stapleton, Ngai, & Moon, 2005b). Interestingly SP5's function in WNT signaling is debated in different contexts, in mouse embryos it acts as a dual activator with β-catenin to fine tune activation of WNT target genes, while in hPSC mediated differentiation it acts as a WNT induced negative regulator of WNT target genes to rein in expression of these genes (Huggins et al., 2017; Kennedy et al., 2016).

The role of SP5 in neural patterning has been partially characterized in Xenopus and murine studies, where knockout of the protein exhibits patterning defects and tail truncations (Dunty et al., 2014a; C. J. Thorpe et al., 2005; Weidinger et al., 2005b). These studies suggest that in neural development, SP5 acts as an activator essential to the formation of the tailbud/posterior neural tube. Here we build on these previous studies to demonstrate the transcriptional role of SP5 in early human neural patterning using our in vitro model of neural development. We show that SP5 is strongly induced in our in vitro model of neural differentiation and patterning, under the direct control of β-catenin binding. Additionally using a previously generated SP5 mutant hPSC line missing the ZF binding domain of SP5 we demonstrate that

neural patterning is impaired midbrain and posterior patterned hNPCs, These results suggest that in human neural patterning SP5 acts as an activator of midbrain and posterior transcriptional programs, potentially in conjunction with β-catenin as has been previously observed in gastrulation events in mouse studies (Kennedy et al., 2016). Finally we show that SP5 binds to its own promoter in the context of neural development, similar previously identified ability to regulate its own expression in hPSCs (Huggins et al., 2017). Future ChIP-seq studies will enable global characterization of SP5 binding during neural patterning in anterior, midbrain, and posterior patterned hNPCs providing mechanistic insight into its transcriptional activity.

These observed results appear to be contradictory to SP5's previously demonstrated action as a negative regulator of WNT target genes (Huggins et al., 2017). However it could be possible that SP5 has evolved to have context specific mechanisms of action, to act as a negative regulator of WNT targets in early pluripotent stem cell differentiation to act as an activator to fine tune WNT target gene activation in the context of neural patterning.

Future work will use ChIP-seq to examine the binding patterns of SP5 during neural patterning, we would hypothesize that SP5 acts similarly to previous observations, with β-catenin at enhancers to fine-tune the activation of WNT target genes required for normal neural patterning. If this is true, the mechanism that deactivates the midbrain specific transcriptional program remains elusive. Potential mechanisms involving miRNA-mediated deactivation are being examined to account for the precise control over this process. Unraveling the concentration dependent negative regulation activity of WNT signaling in this developmental context will enhance our understanding of this pathway, opening up novel therapeutic avenues to treat the myriad of diseases affected by dysregulated aspects of this signaling pathway.

CHAPTER 5

SUMMARY AND FUTURE PERSPECTIVE

5.1. Summary and Future Perspectives

5.1.1. Specific Aim 1: Development and characterization of an in vitro model of neural patterning using human pluripotent stem cells

In this first aim we developed and characterized an in vitro model of neural patterning using human pluripotent stem cells (hPSCs). We generated a WNT responsive reporter line and identified heterogeneity in endogenous WNT signaling in hNPCs as the source of regional identity during the differentiation from hPSCs. These regional identities are stable over long term passaging as apposed to the pluripotent metastable state of hPSCs they were derived from (Cahan & Daley, 2013). These findings corroborate earlier findings from developmental biology studies that demonstrate a gradient of WNT signaling controls the early positional identity of neural progenitors in the neural tube (Kiecker & Niehrs, 2001; Nordström et al., 2002). Other studies have generated regional fates using manipulation of RAs or FGFs (Dimos et al., 2008; D. Yang et al., 2008). We demonstrate that WNT signaling initially specifies regional identity suggesting that additional signals such as RA or FGFs may play roles in further refinement of cell identity. Additionally we demonstrate that exogenous manipulation of WNT signaling during differentiation of hNPCs leads to pure populations of positionally specified neural progenitor cells. These findings can be used to generate neural progenitor cells and neurons with specific regional identities to perform disease modeling or cellular therapies for neurodegerative diseases or injury, which affect specific regions of the adult brain. In summary we develop and characterize a system of human neural patterning using human pluripotent stem cells that demonstrates WNT signaling as controlling the A/P axis of the developing neural tube.

5.1.2. Specific Aim 2: Characterization of the role of β-catenin in regulating fate decisions of human neural progenitor cells in neural patterning

The second aim further utilizes our previously generated model of human neural patterning in human pluripotent stem cells. We investigate the transcriptional mechanisms that control the

generation of positional identity in the developing neural tube. As described previously specific thresholds of β-catenin elicit distinct transcriptional signatures. Graded amounts of WNT signaling elicit unique transcriptional signatures due to β-catenin binding. Furthermore we generated a 3X flag β-catenin hPSC line to characterize β-catenin binding genome wide using ChIP-seq. We found that β-catenin binds mainly unique elements different levels of WNT signaling. We additionally characterized the epigenetic landscape of human neural patterning by computationally identifying enhancer elements using a machine learning based algorithm (Rajagopal et al., 2013). Using this information we found that a majority of β-catenin binding is in enhancer elements. These findings prompt a revision of the model of WNT elicited transcription in which β-catenin binds different context specific enhancer elements at varying concentrations of WNT signaling to specify regional fate of neural progenitor cells. These findings have broader impacts on how we view canonical WNT signaling in the context of cancer development and the generation of efficacious therapeutics to treat diseases caused by aberrant WNT signaling

5.1.3. Specific Aim 3: Characterization of the role of negative regulators of WNT signaling in regulating fate decisions of human neural progenitor cells in neural patterning

The third aim investigated potential mechanisms of negative regulation of WNT signaling in the context of neural development. WNT activation leads to specification of regional identity via activation of specific transcriptional signatures mediated through β-catenin binding. Although we previously demonstrated that β-catenin regulates acquisition of positional cell identity in the developing neural tube, how gene signatures are appropriately turned off at differing thresholds of WNT signaling remained an open question. To investigate how this occurs we decided to examine the role of a WNT induced transcription factor, SP5. The role of SP5 is somewhat controversial with some labs demonstrating an activating effect in concert with β-catenin and other demonstrating it acting as a negative regulator that binds previously activated WNT target genes (Huggins et al., 2017; Kennedy et al., 2016), Developmental biology studies demonstrate a role for SP5 in specifying the posterior development of *Xenopus* embryos with its loss resulting in a severe tail truncation phenotype (Chris J Thorpe, Weidinger, & Moon, 2005). Our work shows

that SP5 is highly expressed in midbrain and posterior patterned hNPCs. Subsequent knockdown of SP5 during neural patterning results in impairment of patterning to midbrain and posterior fates, indicating that it plays a role in activating these fates, potentially in concert with β-catenin. Furthermore I performed initial SP5 ChIP experiments that demonstrate SP5 binds its own promoter, consistent with previous work (Huggins et al., 2017), to regulate its own expression in neural patterning. Further work will be necessary to interrogate the role of SP5 in neural patterning.

5.2. Significance and Contributions

The outcomes of our work have been presented as peer-reviewed journal articles and oral/poster presentations in national and international conferences. Summary of our contributions are listed in below.

1. Moya, N., **Cutts, J**., Gaasterland, T., Willert, K., & Brafman, D. A. (2014). Endogenous WNT Signaling Regulates hPSC-Derived Neural Progenitor Cell Heterogeneity and Specifies Their Regional Identity. Stem Cell Reports, 3(6), 1015–1028.

2. **Cutts J**, Nikkhah M, Brafman DA. (2015) Biomaterial Approaches for Stem Cell-Based Myocardial Tissue Engineering. Biomark. Insights 10(Suppl 1):77-90.

3. **Cutts J**, Brookhouser N, Brafman DA. (2016) Generation of Regionally Specific Neural Progenitor Cells (NPCs) and Neurons from Human Pluripotent Stem Cells (hPSCs). In: Vol ; 2016:121-144.

4. **Cutts J**, Brafman DA. Transcriptional regulation of regional neural fate through WNT/B-catenin signaling. *In preparation.*

Talk/poster conferences:

Engineering Multicellular Self Organization 2017
Investigating the Mechanism of a Multi-State Model of WNT Signaling

Biomedical Engineering Society 2017
Investigating the Mechanism of a Multi-State Model of WNT Signaling

International Society for Stem Cell Research (ISSCR) 2017
Investigating the Mechanism of a Multi-State Model of WNT Signaling

Molecular, Cellular, and Tissue Bioengineering Symposium 2017
Investigating the Mechanism of a Multi-State Model of WNT Signaling

International Society for Stem Cell Research 2016
Investigating the Mechanism of a Multi-State Model of WNT Signaling

Arizona Alzheimer's Consortium 2016
WNT Signaling Specifies Regional Identity of hPSC-derived Neurons

5.3. Significance and Contributions

Few studies have taken into consideration the graded effects of WNT signaling and of these none have investigated the transcriptional mechanism that allows different transcriptional outputs at different levels of WNT signaling. (Kirkeby, Grealish, et al., 2012a; Tiago C. Luis et al., 2011) Here we characterize β-catenin binding in a graded fashion using a model of in vitro neural development we developed. We demonstrate that specific levels of WNT activation lead to different cell fates. We find that in line with previous observations, most β-catenin binding lies within enhancers to influence cell fate, likely through an enhancer promoter looping mechanism (Estarás et al., 2015b; Yochum, 2011; Yochum et al., 2010). Additionally we observe β-catenin binding falls into different classes depending on the presence or absence of peaks at different levels of WNT signaling. Few peaks overlap between graded levels of WNT activation while most are unique to different levels of WNT signaling, corresponding to different hNPC fates. This study expands the traditional binary view of canonical WNT signaling and could help illuminate WNT/β-catenin activity in other developmental and diseased contexts.

Our work focuses mainly on the activating role of WNT signaling in specifying regional identity, how gene programs are appropriately turned off subsequent to WNT stimulated activation remains an open question. There are multiple possibilities regarding how this process could be regulated. One promising mechanism currently under investigation in the lab is the role of mirco rna (miRNA) in relation to canonical WNT signaling which may play a role in turning off M- signature genes. MicroRNAs are small non-coding RNAs that interact with a RNA-induced

silencing complex (RISC) to silence expression of mRNA (Macfarlane & Murphy, 2010). Preliminary findings from this work indicate that mir10a is upregulated in posterior patterned hNPCs. This suggests that this miRNA may play a role as a WNT induced negative regulator of WNT signaling. Of course additional transcription factors from alternative signaling pathways could act to impart the proper transcriptional signature. SP5 is a WNT induced negative regulator of WNT signaling that our preliminary work indicates plays an important role in neural patterning (Huggins et al., 2017). Although our initial work suggests it could play a role in activation further studies that utilize ChIP-seq and overexpression of SP5 in our model of neural development will help interrogate its complete mechanism of action. Additionally, FGFs and RAs are known to play a role in activation of mid- and hindbrain fates and could be responsible for repressing alternative neural progenitor fates, potentially through a WNT mediated mechanism. It's likely that a combination of several of these mechanisms work together to ensure proper patterning on the developing neural tube.

The discovery that cells can self organize has ushered in a new era of developmental biology with the use of cell organoids . Further understanding of the WNT regulated molecular mechanisms involved in neural patterning will enable more accurate engineering of novel neural organoids furthering our ability to model diseases, screen therapeutics, and treat neurodegenerative diseases. This will necessitate novel bioengineering techniques that employ the use of mitogen gradients to properly specify regional fate of neural progenitors in an appropriate spatiotemporal fashion. WNT signaling is fundamentally known to help establish body axis in developing organisms (Loh, van Amerongen, et al., 2016), future bioengineering efforts should take these signals into account.

WNT plays known roles in the genesis of many forms cancer, much work continues to be done to find treatments for WNT caused cancers (B. Chen et al., 2009; Wiese et al., 2018). However our understanding of how WNT elicits transcription continues to evolve. Here we expand the traditional view of WNT elicited transcription using a graded model of WNT signaling. Different thresholds elicit different transcriptional signatures in development of the neural tube. This prompts a reinvestigation of the level of WNT signaling in different types of cancers and

potentially even among different patients with the same cancer. A hypothesis known as the ‘just right hypothesis’ posits that a low level of WNT signaling leads to cancerous phenotype while higher levels lead to apoptosis (Albuquerque et al., 2002). The development of different types of cancers involve the dysregulation of multiple signaling pathways, WNT dysregulation plays a prominent role in the genesis of many types of cnancers. However our results suggest that different levels of WNT could need entirely different treatments because different levels of WNT elicit unique transcriptional signatures. Thus the current state of WNT investigation into cancer should avoid the traditional two state model of WNT signaling in the treatment of cancers affected by WNT signaling. It will be important to take this into consideration, not only for WNT signaling as it is likely this graded mechanisms exit to regulate many different signaling pathways.

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APPENDIX A

SUPPLEMENTARY FIGURES FOR CHAPTER 2

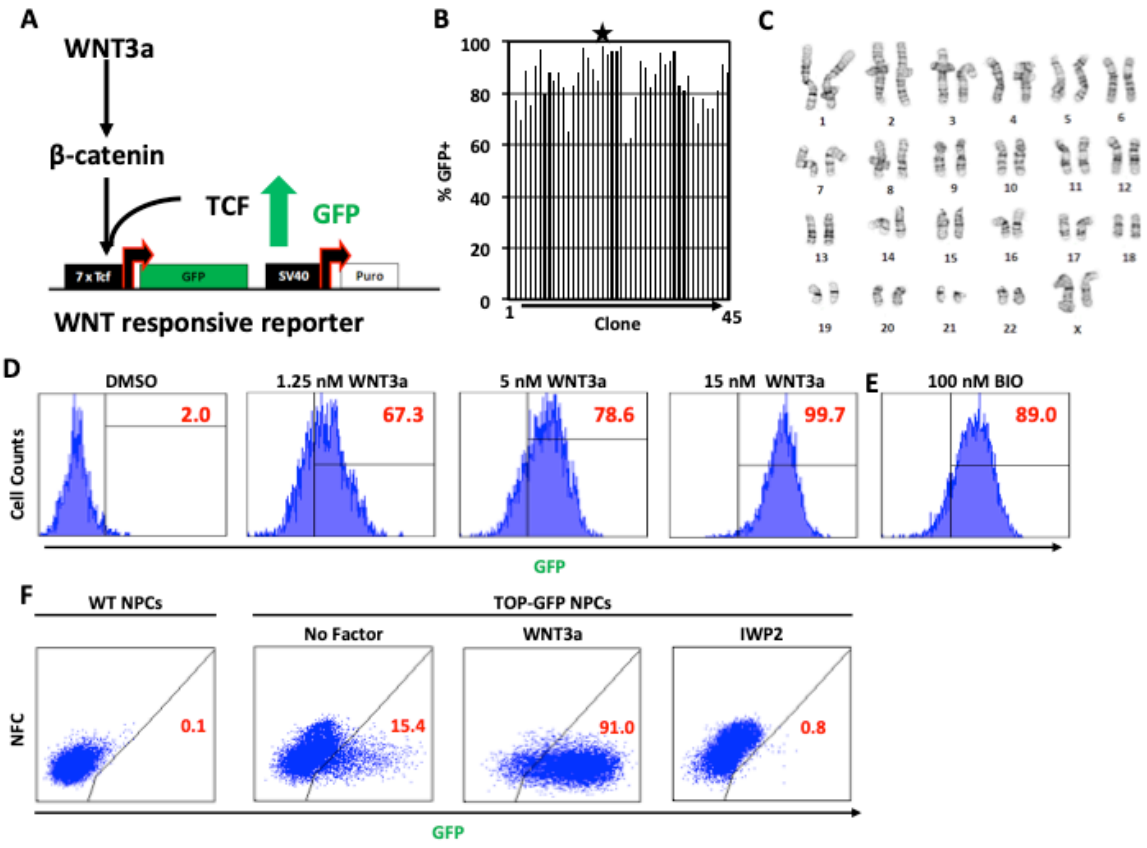

Figure A.1 Generation of clonal WNT reporter hESC lines. A) Schematic of TOP-GFP lentiviral construct. B) Flow cytometry analysis of expanded clones after 48 hour treatment with 15 nM purified mouse WNT3a. Clone 19 (hTOP-19) displayed the highest expression of GFP after WNT3a treatment. C) Karyotype analysis of hTOP-19. Chromosome spread indicated a normal euploid female karyotype (46XX). D) Flow cytometry analysis of hTOP-19 hESCs treated with various concentrations of WNT3a. E) Flow cytometry analysis of hTOP-19 hESCs treated with GSK3β inhibitor BIO. F) Flow cytometry analysis of GFP expression in reporter expressing NPCs after 48 hours of treatment with 15 nM WNT3a or 1000 nM IWP2. Abbreviations: NFC=Non-fluorescing channel.

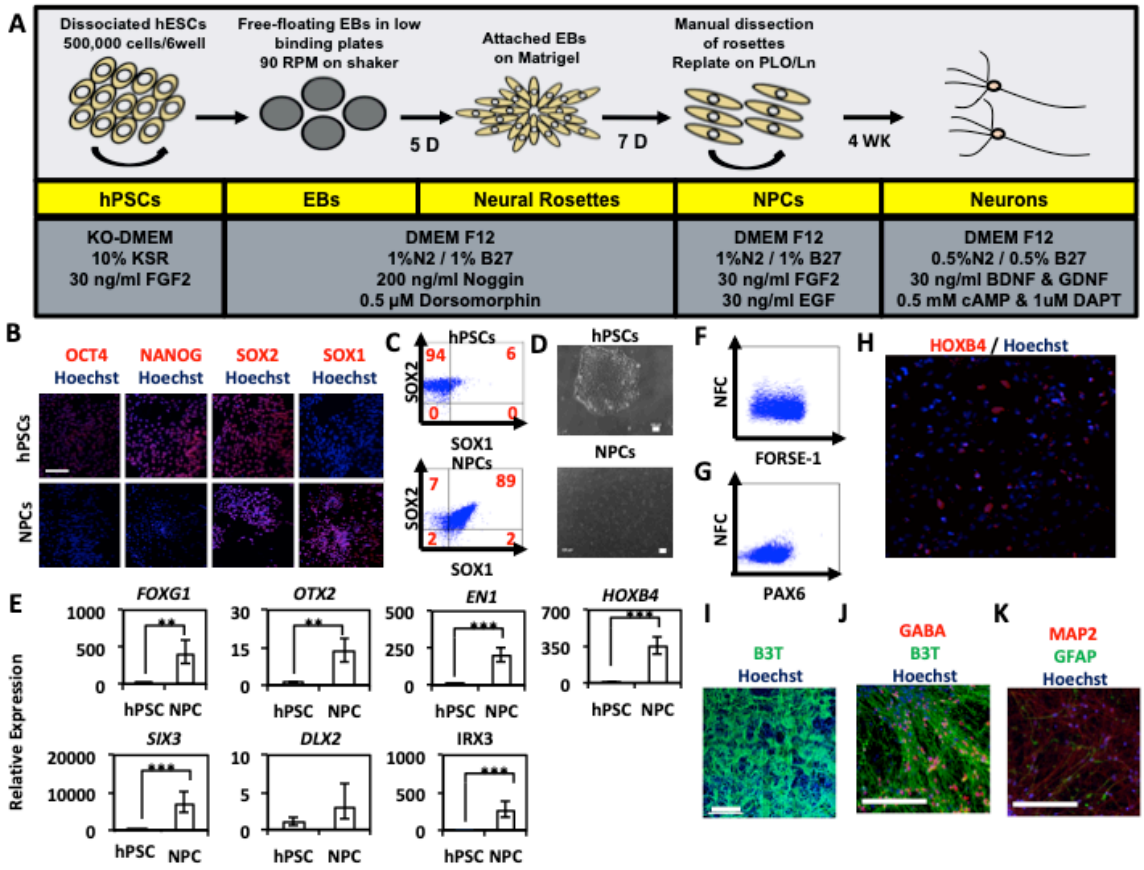

Figure A.2 Differentiation of hPSCs to neural progenitor cells (NPCs) and neurons A) Overview of differentiation protocol for differentiation of hPSCs to NPCs and neruons. The soluble factors, substrate, and culture media at each stage are shown. B) Immunofluorescence of OCT4, NANOG, SOX2, and SOX1 in hESCs and NPCs (scale bar = 100 µm), C) Flow cytometry analysis of SOX1 and SOX2 expression in HESCs and NPCs. Isotype controls used are listed in Table S2 D) Phase contrast images of hESCs and NPCs (scale bar = 100 µm). E) Gene expression analysis of anterior/posterior (A/P) neural tube related genes in hESCs and NPCs (mean ± S.E.M., n=3 independent experiments). Populations were compared using Student's t-test. *p<0.05, **p<0.01, ***p<0.001. Flow cytometry analysis of F) FORSE-1 and G) PAX6 in NPCs H) Immunofluorescence of HOXB4 in NPCs (scale bar = 200 µm). I) Immunofluorescence of B3T in neuronal cultures (scale bar = 1 mm). J) Immunofluorescence of MAP2 and GFAP in neuronal cultures (scale bar = 200 µm). K) B3T and γ-Aminobutyric acid (GABA) in neurons differentiated from hESCs (scale bar = 200 µm). Abbreviations: NFC=Non-fluorescing channel.

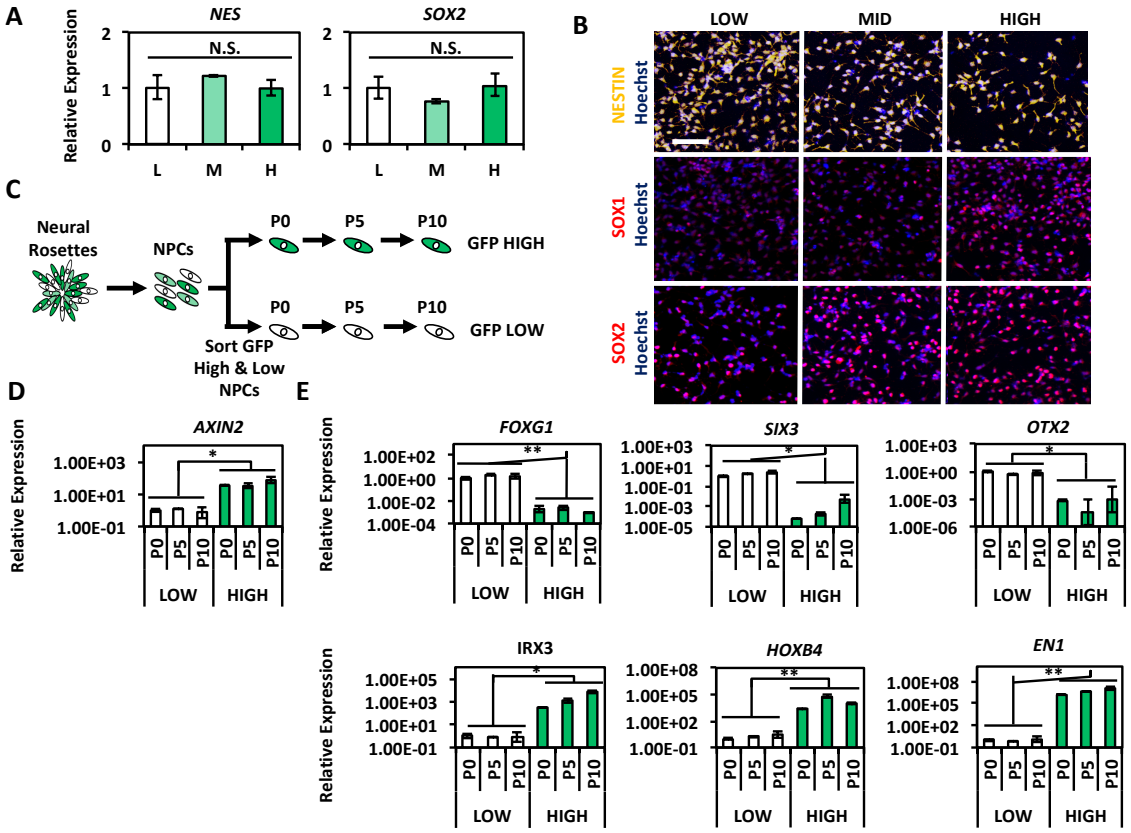

Figure A.3 Analysis of TOP-GFP expressing NPC populations A) Gene expression analysis of SOX2 and NESTIN (mean ± S.E.M., n=3 independent experiments). B) Immunofluorescence of NESTIN, SOX1, and SOX2 C) Reporter expressing NPCs were separated by fluorescence-based cell sorting into GFP^{HIGH} and GFP^{LOW} populations on the basis of GFP expression. GFP^{HIGH} and GFP^{LOW} populations were subsequently cultures for 10 passages (>50 days) and examined for expression of WNT and A/P related genes. D) Gene expression of WNT target gene AXIN2 in GFP sorted NPC populations after 5 and 10 passages(mean ± S.E.M., n=3 independent experiments). E) Gene expression analysis of A/P related genes in GFP sorted NPC populations after 5 and 10 passages (mean ± S.E.M., n=3 independent experiments). Populations were compared using Student's t-test. *p<0.05, **p<0.01, ***p<0.001. Abbreviations N.S. = Not statistically significant

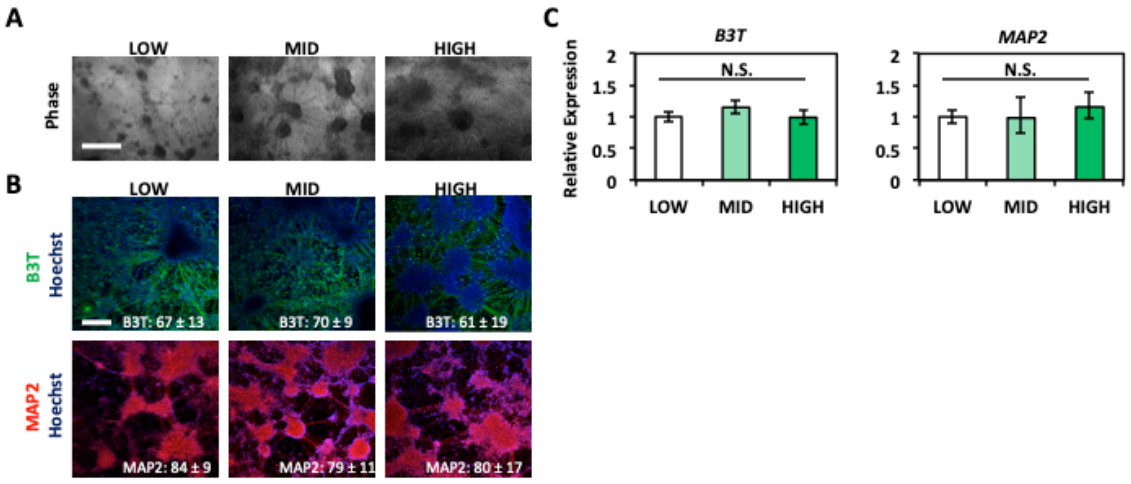

Figure A.4 Analysis of neurons generated from TOP-GFP expressing NPC populations A) Phase contrast images of neurons derived from sorted GFP expressing NPC populations (scale bar = 500 μm). B) Immunofluorescence of mature neuronal markers MAP2 and B3T in neuronal cultures differentiated from sorted GFP^{LOW}, GFP^{MID}, and GFP^{HIGH} NPC populations (scale bar = 200 μm). C) Gene expression analysis of MAP2 and B3T in neuronal cultures differentiated from sorted GFP^{LOW}, GFP^{MID}, and GFP^{HIGH} NPC populations (mean ± S.E.M., n=3 independent experiments). Populations were compared using Student's t-test. *p<0.05, **p<0.01, ***p<0.001. Abbreviations N.S. = Not statistically significant

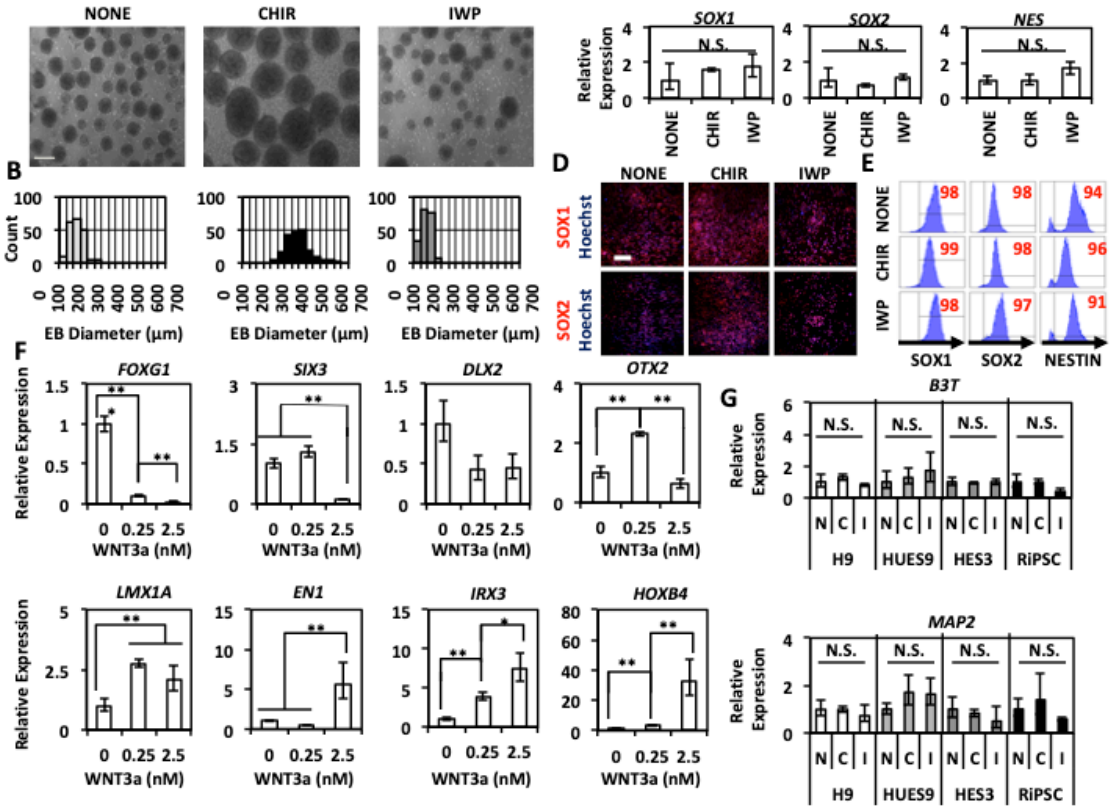

Figure A.5 Analysis of CHIR-, WNT-, IWP2-, and un-treated embryoid bodies, NPCs, and neurons. A) Phase contrast images and size distribution of embryoid bodies (EBs) generated in 500 nM CHIR-, 1000 nM IWP2, and un-treated conditions (scale bar = 200 µm). B) Size distribution of EBs generated in 500 nM CHIR-, 1000 nM IWP2-, and un-treated conditions. The diameter of 200 EBs was measured for each condition. C) Gene expression (mean ± S.E.M., n=3 independent experiments), D) Immunofluorescence (scale bar = 100 µm), and E) flow cytometry analysis of NESTIN, SOX1, and SOX2 of NPCs generated in 500 nM CHIR-, 1000 nM IWP2-, and un-treated conditions. Isotype controls used are listed in Table S2 F) Gene expression analysis of anterior/posterior (A/P) neural tube related genes in NPCs (mean ± S.E.M., n=3 independent experiments) generated in the presence of various WNT concentrations. Populations were compared using Student's t-test. *p<0.05, **p<0.01, ***p<0.001. G) Gene expression analysis of MAP2 and B3T in neuronal cultures differentiated from NPCs generated in 500 nM CHIR-, 1000 nM IWP2-, and un-treated conditions (mean ± S.E.M., n=4 independent

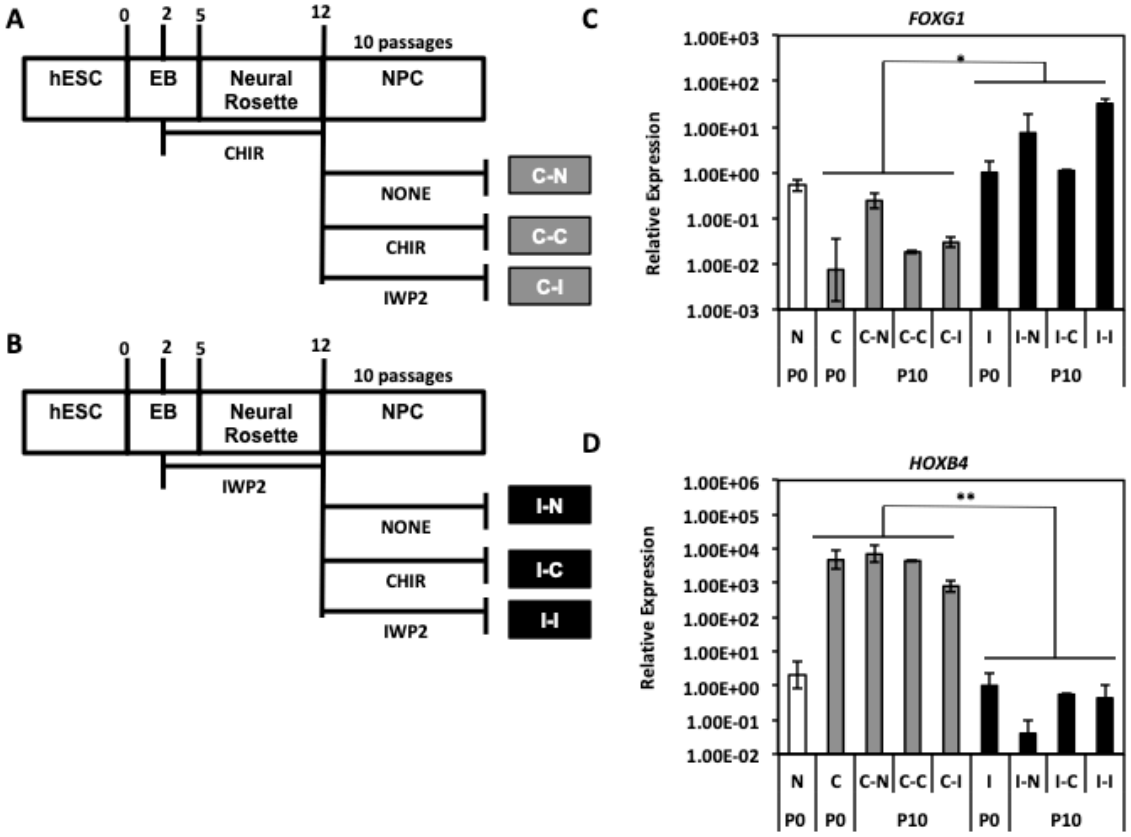

Figure A.6 Analysis of stability of patterning of NPCs imposed by exogenous WNT manipulation A) Posterior-patterned NPCs (i.e. NPCs generated in the presence of 500 nM CHIR) were cultures without CHIR (C-N) or in the presence of 500 nM CHIR (C-C) or 1000 nM IWP2 (C-I) for 10 passages. B) Anterior-patterned NPCs (i.e. NPCs generated in the presence of 1000 nM IWP2 were cultures without IWP2 (I-N) or in the presence of 500 nM CHIR (I-C) or 1000 nM IWP2 (I-I) for 10 passages. Expression of C) FOXG1 and D) HOXB4 was assessed in all conditions after 10 passages and compared to initial passage (P0) NPC cultures (mean ± S.E.M., n=3 independent experiments) . Populations were compared using Student's t-test. *p<0.05, **p<0.01, ***p<0.001.

Table A. 1 TaqMan gene expression assays used in this study.

Gene	ABI Assay
18s	Hs99999901_s1
AXIN2	Hs00610344_m1
CTIP2 (BCL11B)	Hs00256257_m1
CHAT	Hs00252848_m1
CUX1	Hs00738851_m1
DLX2	Hs00269993_m1
EMX1	Hs00417957_m1
EN1	Hs00154977_m1
FGF5	Hs03676587_s1
FOXG1	Hs01850784_s1
GABRA1	Hs00168058_m1
GATA2	Hs00231119_m1
GATA3	Hs00231122_m1
GFAP	Hs00157674_m1
HOXB4	Hs00256884-m1
HOXB6	Hs00980016_m1
IRX3	Hs00735523_m1
LMX1A	Hs00892663_m1
LMX1B	Hs00158750_m1
MAP2	Hs00258900_m1
MNX1 (HB9)	Hs00907365_m1
NES	Hs00707120_s1
NURR1 (NR4A2)	Hs00428691_m1
OTX2	Hs00222238-m1
PITX3	Hs01013935_g1
SATB2	Hs00392652_m1
SIX3	Hs00193667_m1
SOX1	HS01057642_s1
SOX2	Hs01053049_s1
SP5	Hs01370227_mH
TH	Hs00165941_m1
TUBB3	Hs00801390_s1

Table A 2 Antibodies used in this study

Antibody	Vendor	Catalog #	Concentration Used
Goat anti-SOX2	Santa Cruz	SC-17320	1:50
Goat anti-OTX2	R&D Systems	AF1979	1:200
Mouse anti-B3T	Fitzgerald	10R-T136A	1:1000
Mouse anti-EN1	DSHB	4G11	1:800
Mouse anti-FORSE-1	DSHB	Concentrate	1:75
Mouse anti-GFAP	Millipore	AB360	1:500
Mouse anti-MNX1	DSHB	81.5C10	1:100
Mouse anti-Nestin	BD	560341	1:10
Mouse anti-SOX1	BD	560749	1:10
Rabbit anti-FOXG1	Abcam	AB18259	1:100
Rabbit anti-GABA	Millipore	AB15415	1:200
Rabbit anti-HOXA2	Sigma	HPA029774	1:200
Rabbit anti-HOXB4	Abcam	AB76093	1:10
Rabbit anti-LMX1A	Abcam	AB139726	1:100
Rabbit anti-MAP2	Millipore	AB5622	1:500
Rabbit anti-NANOG	Santa Cruz	SC-33759	1:50
Rabbit anti-NURR1	Millipore	AB5778	1:200
Rabbit anti-OCT4	Santa Cruz	SC-9081	1:50
Rabbit anti-TBR1	Abcam	AB31940	1:200
Alexa-647 Mouse Anti-SOX2	BD	560294	20 µl per test
PE Mouse anti-Nestin	BD	561230	5 µl per test
PE Mouse anti-PAX6	BD	561552	5 µl per test
PerCp-Cy5.5 Mouse anti-SOX1	BD	561549	5 µl per test
Alexa-647 Mouse IgG2a Isotype Control	BD	558053	20 µl per test
PE Mouse IgG1 Isotype Control	BioLegend	400113	5 ul per test
PE Mouse IgG2a Isotype Control	BD	561552	5 ul per test
PercCp-Cy5.5 MouseIgG1 Isotype Control	BD	550795	5 ul per test
Alexa 647 Donkey Anti-Goat	Life Technologies	A-21447	1:200
Alexa 647 Donkey Anti-Rabbit	Life Technologies	A-31573	1:200
Alexa 647 Donkey Anti-Mouse	Life Technologies	A-31571	1:200
Alexa 546 Donkey Anti-Goat	Life Technologies	A-11056	1:200
Alexa 546 Donkey Anti-Rabbit	Life Technologies	A-10040	1:200
Alexa 546 Donkey Anti-Mouse	Life Technologies	A-10036	1:200
Alexa 488 Donkey Anti-Goat	Life Technologies	A-11055	1:200
Alexa 488 Donkey Anti-Rabbit	Life Technologies	A-21206	1:200
Alexa 488 Donkey Anti-Mouse	Life Technologies	A-21202	1:200

APPENDIX B

SUPPLEMENTARY FIGURES FOR CHAPTER 3

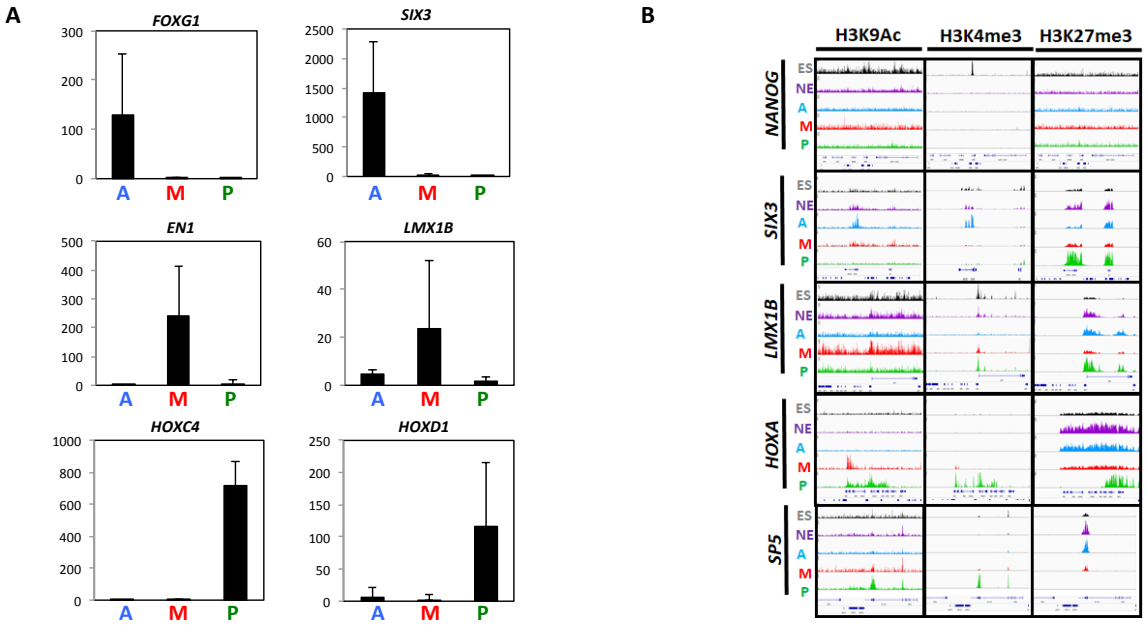

Figure B.1 Validation of characterization of in vitro model of neural patterning A) Gene-expression analysis of A/P related genes in A/P patterned hNPCs (mean ± SEM, n=3 independent experiments) B) Genome browser captures of representative genes for H3K9ac, H3K4me3, and H3K27ac in respective gene promoters

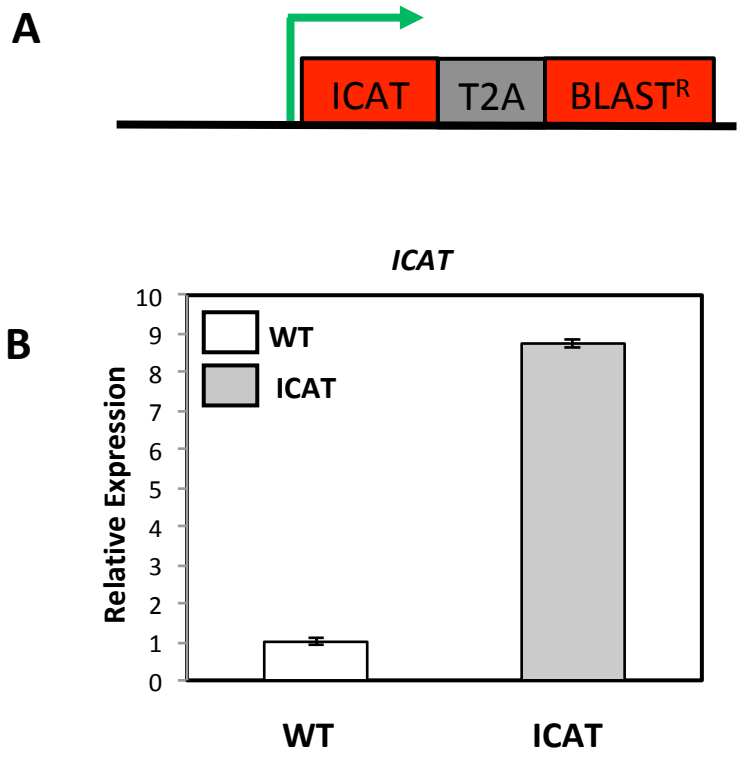

Figure B.2 Generation and characterization of ICAT overexpression embryonic stem cells A) Schematic of ICAT overexpression vector B) Gene-expression analysis of ICAT in H1 WT and ICAT overexpression (mean ± SEM, n=3 independent experiments)

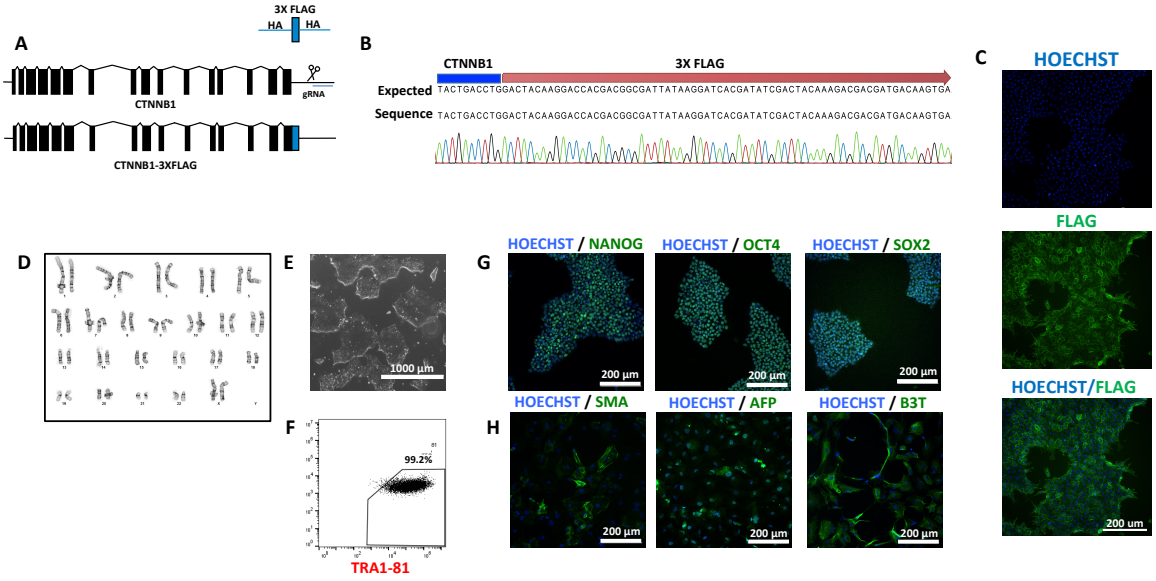

Figure B.3 Generation and characterization of CTNNB1-3XFLAG embryonic stem cells A) Schematic of CRISPR mediated strategy to insert 3X FLAG at C-terminus of CTNNB1 B) Sequencing of selected CTNNB1-3XFLAG clones C) Fluorescent images of α-FLAG staining of CTNNB1-3XFLAG line D) Karyotype analysis of CTNNB1-3X FLAG line E) Phase contrast image D) Flow cytometry analysis of pluripotency cell surface marker TRA1-81 G) Fluorescent images of pluripotency markers NANOG, OCT4, and SOX2 H) Fluorescent images of CTNNB1-3XFLAG cells subjected to undirected differentiation and analyzed for genes representative of germ layers SMA, AFP, and B3T.

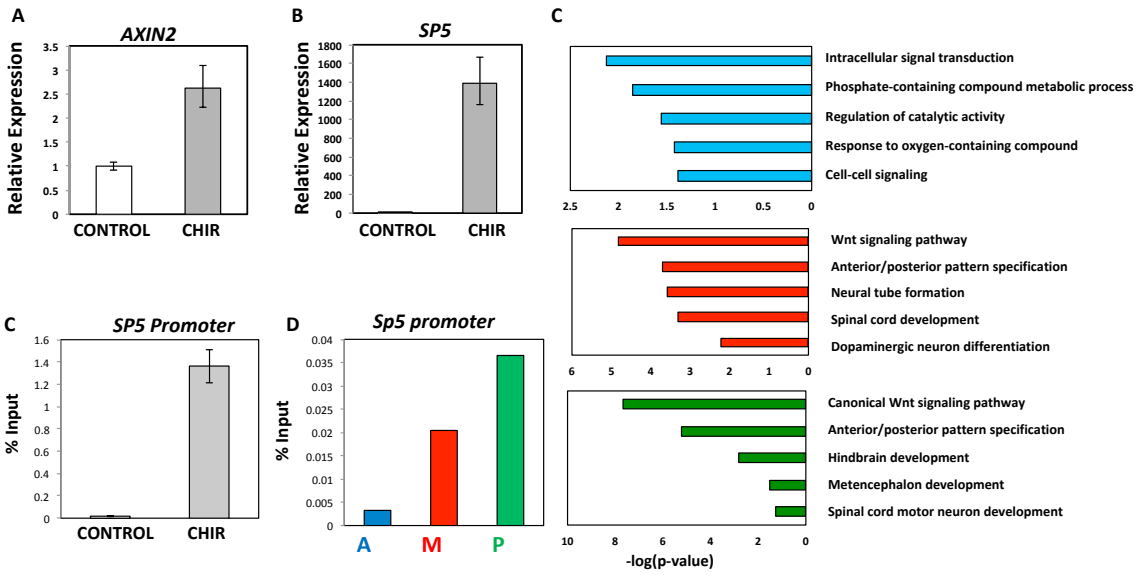

Figure B.4 Analysis of β-catenin identified peaks A) Gene expression analysis of WNT targets AXIN2 and B) SP5 in CTNNB1-3XFLAG embryonic stem cells treated with or without 0.5 μm CHIR98014 for 48 hours (mean ± SEM, n=3 independent experiments) C) ChIP-qPCR of SP5 promoter in CTNNB1-3XFLAG embryonic stem cells treated with or without 0.5 μm CHIR98014 for 48 hours (mean ± SEM, n=3 independent experiments) D) ChIP-qPCR of SP5 promoter in CTNNB1-3XFLAG embryonic stem cells patterned to Anterior (A), Midbrain (M), or Posterior (P) patterned hNPCs (mean ± SEM, n=3 independent experiments E) Gene ontology analysis of genes regulated by β-catenin peaks identified in Anterior (A), Midbrain (M), and Posterior (P) patterned hNPCs.

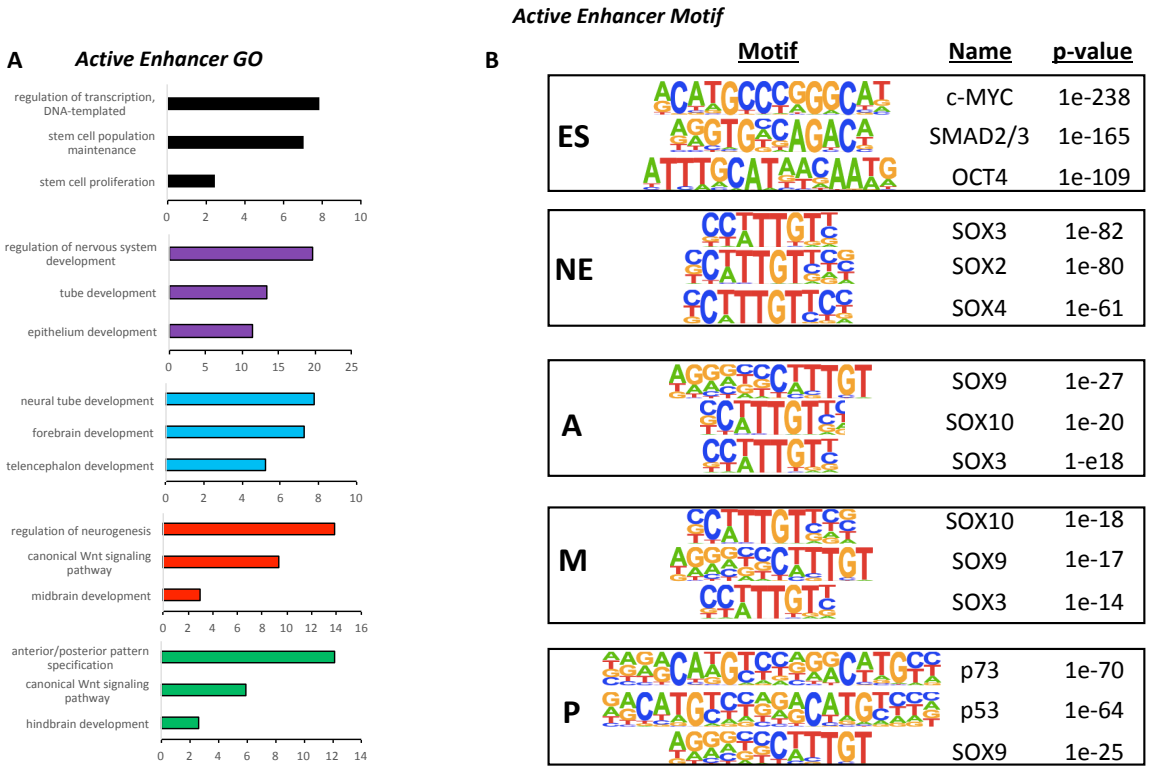

Figure B.5 Analysis of Enhancers identified during neural differentiation and patterning A) Gene ontology analysis of genes linked to active enhancers B) Motif analysis of active enhancers identified during neural differentiation and patterning

APPENDIX C

CO-AUTHOR PERMISSIONS

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Title: Endogenous WNT Signaling Regulates hPSC-Derived Neural Progenitor Cell Heterogeneity and Specifies Their Regional Identity

Author: Noel Moya,Josh Cutts,Terry Gaasterland,Karl Willert,David A. Brafman

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