NOS1APc ASSOCIATES WITH HIPPO SIGNALING COMPONENTS AND CONTRIBUTES TO SPINAL CORD DEVELOPMENT

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Abstract

The nitric oxide synthase 1 adaptor protein (NOS1AP) is an adaptor protein implicated in a number of human conditions including schizophrenia, anxiety and cardiac QT syndrome. Previous studies have shown that NOS1AP and some of its isoforms associate with the tumor suppressor protein scribble. Since scribble has been linked to the Hippo pathway, I set out to determine if NOS1AP associates with the Hippo pathway and whether it controls aspects of neuronal development.

Here I show that NOS1AP and NOS1APc interact with the transcriptional co-activator yes-associated protein (YAP), a component of the Hippo cascade. Further both NOS1APa and NOS1APc show partial co-distribute with YAP in HEK293Tcells, with NOS1APc having better co-distribution. *In situ* hybridization and immunocytochemistry studies reveal that NOS1APc is expressed in the developing spinal cord. NOS1APc is expressed in the floor plate and roof plate and shows a similar profile to radial glial cells. *In ovo* electroporation of cDNA constructs encoding NOS1APc in the developing spinal cord induced ectopic SOX2+ rosettes in the marginal zone similar to that seen with Yap overexpression, implicating NOS1APc in cellular proliferation.

Taken together, these data suggests NOS1APc plays a role in the developing spinal cord, acting through the Hippo signaling pathway to affect neural progenitor cellular differentiation.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ActR2a	Actin-related protein 2a homolog
ActR2b	Actin-related protein 2b homolog
AP	Anterior-posterior
APC	Adenomatous polyposis coli
aPKC	Atypical protein kinase C
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bHLH	Basic helix-loop-helix
BMPR1a	Bone morphogenetic protein receptor type I a
BMPR1b	Bone morphogenetic protein receptor type I b
BMPR2	Bone morphogenetic protein receptor type II
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAPON	Carboxy terminal PDZ ligand of nNOS
cDNA	Complementary DNA
CNS	Central nervous system
Crb	Crumb
C-terminal	Carboxyl-terminal
Dchs1	Dachsous Cadherin-Related 1
Dchs2	Dachsous Cadherin-Related 2
Dco	Discs overgrown
ddH_2O	Double-distilled water
Dlg	Discs-large
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
Dvl	Dishevelled
EMT	Epithelial-mesenchymal transition
Ex1	Expanded-1
Ex2	Expanded-2
Fat4/FatJ	Fat homolog in mammals
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Fj	Four-jointed
Fjx1	Human homolog of four-jointed
FP	Floor Plate
FRMD1	FERM domain containing 1
FRMD6	FERM domain containing 6
GFP	Green Fluorescent Protein
GliA	Activator form of Gli
GliR	Repressor form of Gli
GSK-3	Glycogen synthase kinase 3
GSK3β	Glycogen synthase kinase 3 beta
HD	Homeodomain

HEK	Human Embryonic Kidney
HOX	Homeotic genes
HPO	Hippo
IHC	Immunohistochemistry
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinases
KIBRA	Kidney/BRAin protein
LAP	Latency associated peptide
Lats1	Large Tumour Suppressor 1
Lats2	Large Tumour Suppressor 2
Lft	Lowfat
Lgl	Lethal-giant larvae
Lix1	Lowfat human homolog
Lix1L	Lix1-like protein
LPR5	Low-density lipoprotein receptor-related protein 5
LPR6	Low-density lipoprotein receptor-related protein 6
LRR	Leucine-rich repeat
Mats	Mod-as-tumor suppressor
MCF7	Michigan Cancer Foundation-7, a breast cancer cell line
Mer	Merlin
mRNA	Messenger RNA
Mst1/Mst2	Mammalian hippo homologs
Mob1	MOBKL1A and MOBKL1B, collectively. Two Mats mammalian homologs.
MOBKL1A	Mps one binder kinase activator-like 1A
MOBKL1B	Mps one binder kinase activator-like 1b
NBT	Nitro blue tetrazolium chloride
NEC	Neuroepithelial Cells
NF2	Human homolog of Merlin
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
NOS1AP	Nitric oxide synthase 1 adaptor protein
N-terminal	Amino-terminal
OCT	Optimum cutting temperature
PBS	Phosphate buffer saline
PCP	Planar cell polarity
PDZ	Post-synaptic density 95, PSD-95; discs large, Dlg; zonlua occludens-1, ZO-1 binding motif
PEI	Polyethylenimine
PFA	Paraformaldehyde
PKA	Protein Kinase A
PMSF	Phenylmethylsulphonyl fluoride
PNS	Peripheral nervous system
PTB	Phosphotyrosine binding domain
pYAP	Phosphorylated yes associated protein
R-Smad	Receptor-regulated SMADs

RA	Retinoic Acid
RGCs	Radial glial cells
RNA	Ribonucleic Acid
RT	Room temperature
Runx2	Runt related transcription factor 2
Sav	Salvador
Sav1	Mammalian Salvador homolog
SB	Sample buffer
Scrib	Scribble
Sd	Scalloped
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
Shh	Sonic Hedgehog
Ski	Skinny Hedgehog
SMO	Smoothened
SOX2	SRY(sex determining region Y)-box 2
SSC	Saline sodium citrate
Stbm	Strabismus
Sufu	Suppressor of Fused
TAZ	Tafazzin
TBST	Tris-buffer saline tween
TCF	T-Cell specific
TEAD/TEF	TEA domain-containing transcription factor
TGFβ	Transforming growth factor family
Wts	Warts
WWC1	WW-domain containing protein 1
WWC2	WW-domain containing protein 2
YAP	Yes Association Protein
YFP	Yellow fluorescent protein
Yki	Yorkie

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CHAPTER 1 INTRODUCTION

There are many different types of unique movements observed in vertebrates; one prevailing motion observed consistently across the vertebrate population is locomotion. Locomotion, such as walking, swimming or flying, is an important, fundamental component of animal behavior (Grillner, 1975, Dickinson et al., 2000). Though it appears kinetically as a fundamental functional task, this motor task in vertebrates is accomplished through a complex network, which integrates different neural circuits and signaling pathways required to accurately activate muscles in precise temporal and spatial patterns controlled by the spinal cord (Ladle et al., 2007, Goulding, 2009, Jung and Dasen, 2015).

Due to its fundamental and repetitive nature, locomotion, more specifically walking, has become a focal point of study and has proven to be an excellent model in gaining insight into the development and organization of the spinal cord and the many pathways that are integrated. (Kiehn, 2011, Lacquaniti et al., 2012).

1.1 NEURONAL INDUCTION

In vertebrates, the central nervous system (CNS) is known to develop from the ectoderm (Chang and Hemmati-Brivanlou, 1998). Studies performed by Spemann and Mangold using amphibian embryos showed that in order to generate a properly patterned nervous system, inductive signals released by the dorsal lip of the blastopore, a region called the organizer was necessary to begin neural induction (Stern, 2005, Gouti et al., 2015). At the onset of gastrulation, the mesodermal cells

migrate through the dorsal blastopore lip and form a layer in between the endoderm and the ectoderm. These mesodermally derived cells, known as axial mesoderm, then migrate along the dorsal midline and give rise to a structure called the notochord (Ozair et al., 2013). The notochord is known to secrete specific signaling molecules that induce the formation of neuroepithelial cells. These signals are known as neural inducers; they include bone-morphogenetic protein antagonists, such as Chordin, Noggin, Follistatin, and Nodal, which function to inhibit the activity of the bone-morphogenetic signaling pathway (BMP pathway) (Harland, 2000). The BMP pathway is known to induce epidermal cell differentiation during development (Munoz-Sanjuan and Brivanlou, 2002, Copp et al., 2003).

After neural induction, the dorsal ectoderm has become neuroepithelial cells, which dorsally condense to form the neural plate (Harland, 2000). The neural plate then develops bilateral neural folds at its junction with surface (non-neural) ectoderm (Geelen and Langman, 1979, Copp et al., 2003). These folds elevate, come into contact in the midline, and fuse to create the neural tube. As the neural plate folds and closes, specific cells known as neural crest cells detach from its lateral margins and migrate away, later condensing to form the major part of the peripheral nervous system (PNS). As development proceeds, the neural tube will then become covered in epidermal ectoderm (Geelen and Langman, 1979, Copp et al., 2003).

Neurulation, which functions to produce the brain and spinal cord, concludes with the formation of the neural tube, by the closure of the posterior neuropore at the caudal end of the neural tube. The ventral part of the neural tube is called the basal plate. Dorsal part is called the alar plate. The hollow interior is called the neural canal (Copp et al., 2003).

1.2 NEUROGENESIS

The spinal cord continues to develop after the closure of the neural tube. As the spinal cord develops specific dorsal (roof plate) and ventral (floor plate) regions of the neural tube are necessary for the dorsoventral patterning of the neural tube (Jessell, 2000). Three classes of signaling molecules play prominent roles in specifying distinct cell types along the dorsoventral axis of the spinal cord. Sonic hedgehog (Shh), bone morphogenetic (BMPs) and Wnt proteins have emerged as the primary inductive signals of neuronal progenitor cell specification. Shh acts ventrally and BMP and Wnt act dorsally (Jessell, 2000). The ability of these progenitor cells to differentiate these inductive signals largely involves alterations in gene expression of regulatory transcription factors, such as homeodomain (HD) and basic helix-loop-helix (bHLH) factors (Jessell, 2000).

Typically, cell division in the developing neural tube takes place in the ventricular zone surrounding the central canal. In the beginning stages of spinal cord development, the neuroepithelial cells (NEC) undergo a period of symmetric proliferative divisions, forming the neuroepithelium of the neural tube (Paridaen

and Huttner, 2014). These cells span the entire neuroepithelium and extend processes to the apical ventricular lumen and the basal pial surface. The ventricular zone is the most apical layer of the neuroepithelium that contains most of the progenitor cell bodies (Paridaen and Huttner, 2014). Neural progenitor cells can be detected by a common transcription factor, SOX2, which belongs to the Src gene family. SOX2 is known for promoting cell proliferation and is only expressed in cells undergoing proliferative division (Paridaen and Huttner, 2014).

In the majority of the defined rostral areas of the neural tube, which eventually develop into the different brain regions, the onset of neurogenesis causes NEC to switch their identity and become radial glial cells (RGCs), also known as progenitor cells, by down-regulating epithelial features such as Golgi-derived apical trafficking and losing tight junctions while maintaining adherens junctions. This is detected in mice between embryonic day E10 and E11 (Gotz and Huttner, 2005, Knoblich, 2008).

Interestingly, neural progenitors undergoing neurogenesis in the retina and spinal cord are thought to differ from those residing in the rest of the CNS regions in that they maintain NEC properties during neurogenesis, while also possessing a broader developmental potential throughout neurogenesis. This allows for the development of specific progenitor domains to form along the dorsoventral axis of the spinal cord (Gotz and Huttner, 2005).

At the onset of neurogenesis, these symmetrically dividing neural progenitor cells switch to the majority of them dividing asymmetrically yielding one progenitor daughter cell and one differentiating daughter cell (Dessaud et al., 2008, Knoblich, 2008, Paridaen and Huttner, 2014). The apical-basal polarity of these neural progenitor cells plays an important role in the determination of symmetric versus asymmetric division. It has been proposed that cleavage planes that are oriented in the radial dimension of the ventricular zone result in symmetric, proliferative divisions of neural progenitor cells due to equal distribution of cellular contents (Dessaud et al., 2008, Paridaen and Huttner, 2014). Asymmetric division relies on the unequal distribution of cell components that determine cell fate. An active reorientation of the mitotic spindle into an apical-basal orientation is responsible for the asymmetric vertebrate neural progenitor division (Dessaud et al., 2008, Knoblich, 2008, Paridaen and Huttner, 2014). At this point, the differentiating daughter cell receives a combination of extracellular signals that allow for the activation or repression of specific transcription factors allowing it to either develop into a neuron or later in development, a glial cell (Gotz and Huttner, 2005, Knoblich, 2008, Paridaen and Huttner, 2014).

As stated previously, it has been shown that two classes of signaling molecules play prominent roles in specifying distinct cell types along the dorsoventral axis of the spinal cord. The two identified are members of the transforming growth factor (TGFβ) superfamily acting dorsally and Sonic hedgehog acting ventrally. Dividing progenitor cells within the ventricular zone respond via expressed surface

membrane receptors to the types and concentrations of TGFβs and Shh in order to determine their position as they become postmitotic and migrate laterally into the mantle region (Fig: 1.1).

1.3 VENTRAL SPINAL CORD CELL FATE SPECIFICATION

Sonic hedgehog is the main signaling molecule responsible for the ventral patterning of progenitor domains in the developing neural tube, acting through the Hedgehog Signaling Pathway (Briscoe and Ericson, 1999, Jessell, 2000). In the earliest stages of neurogenesis, the ventrally located notochord is the primary source of graded Shh expression (Teillet et al., 1998, Patten and Placzek, 2000). Shh is known to induce the ventral neural tube formation including the floor plate (FP). In the later stages, the notochord regresses below the ventral neural tube and the FP becomes the major source of localized Shh gradient (Teillet et al., 1998, Patten and Placzek, 2000). Shh is secreted in a ventral high, dorsal low gradient from the floor plate cells and gives rise to six well-established progenitor domains pMN, p3, p2, p1, and p0, respectively (Briscoe and Ericson, 1999, Jessell, 2000, Briscoe and Ericson, 2001). Each progenitor domain gives rise to one or more specific spinal cord cell types necessary for the development of spinal networks that give rise to normal gait (Jessell, 2000). Networks of the HD family members and the bHLH family members of transcriptional factors expressed on ventral neural progenitor cells drive the creation of five post-mitotic differentiated neuron populations. These transcriptional factors are expressed in response to Shh levels, as well as the

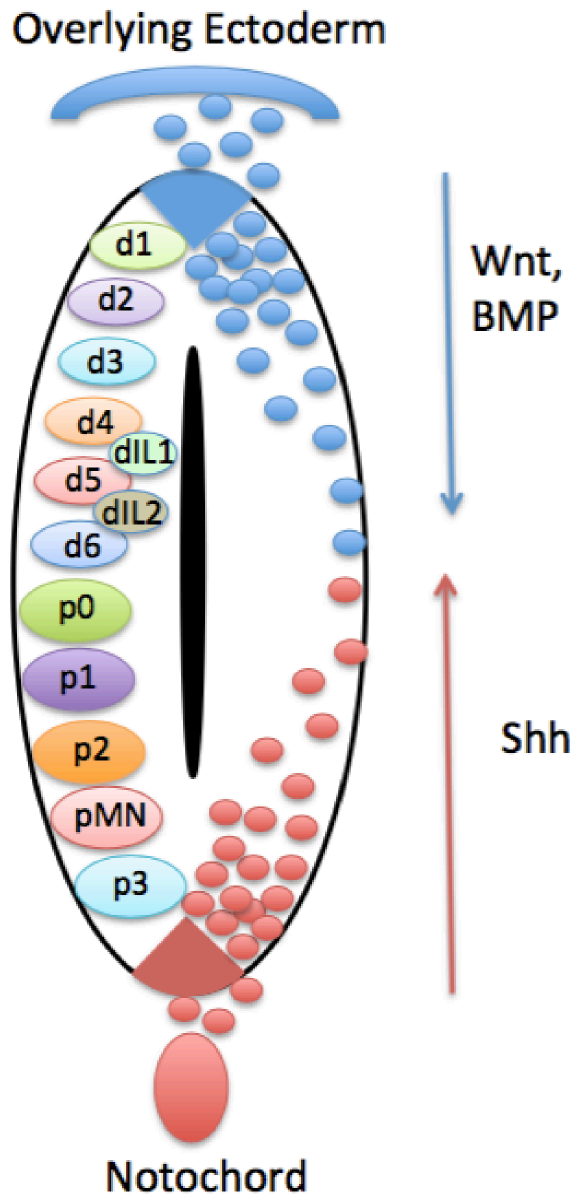

Figure 1.1: Dorsoventral Patterning of the Neural Tube: Shh (red) released from the floor plate (FP) and Wnt/BMP (blue) released from the roof plate (RP) are responsible for the concentration dependent dorsal-ventral neural tube progenitor domain patterning seen on the left. These progenitor domains eventually produce specific differentiated neurons. (Figure modified from Aviles et al. 2013).

duration for which they are exposed to Shh. (Briscoe and Ericson, 1999, Jessell, 2000, Briscoe and Ericson, Dessaud et al., 2008, Cohen et al., 2013).

1.3.1 SHH Signaling in Ventral Spinal Cord Cell Specification

Before Shh is secreted, it undergoes a series of post-translational modifications, which involves dual lipidation by cholesterol and palmitic acid (Lee et al., 1994, Bumcrot et al., 1995, Pepinsky et al., 1998). The active form of Shh is cholesterol modified at the c-terminus, which helps in the secretory regulation and the long-range distribution and activity of Shh. Shh is also palmitoylated at the N-terminus, which is speculated to be added by Skinny Hedgehog (ski), an acyltransferase. The pamitoyl group is known to increase the activation abilities of Shh (Pepinsky et al., 1998, Dessaud et al., 2008, Briscoe and Therond, 2013).

This dual lipidation process is believed to allow Shh to interact with Dispatched, a 12-pass transmembrane transport protein that contains a sterol-sensing domain (SSD) in order to be released from the plasma membrane of the secretory cell (Caspary et al., 2002, Dessaud et al., 2008, Briscoe and Therond, 2013). Shh is then thought to interact with a number of surface proteins, decreasing its ability to spread dorsally in a gradient manner. These surface proteins are also responsible for enhancing or inhibiting the signaling ability of Shh in a cell autonomous manner (Dessaud et al., 2008).

Recently the primary cilium of the neural progenitor cells has been identified to be necessary for the transduction of Shh signaling (Eggenschwiler and Anderson, 2007, Rohatgi et al., 2007, Ruat et al., 2012). As Shh moves dorsally, receptors located within the primary cilium membrane integrate the Shh levels, which eventually leads to neuronal differentiation. Shh is secreted in a concentration gradient manner, with the highest concentration being observed in the most ventral progenitors domains, decreasing as you move dorsally through the ventral progenitor domains (Briscoe and Ericson, 1999, Jessell, 2000, Briscoe and Ericson, 2001, Dessaud et al., 2008). It is observed that increasing and sustained concentrations of Shh allow longer maintenance of the intracellular Gli signaling activity, which results in progenitor cells differentiating into more ventral identities (Dessaud et al., 2008, Ruat et al., 2012).

The Shh signaling transduction pathway ultimately affects three transcription factors of the Gli family, Gli1, Gli2, and Gli3. In the absence of Shh, smoothened (SMO) is inhibited by a membrane bound receptor patched (Ptc), known as the binding receptor for Shh (Dessaud et al., 2008, Briscoe and Therond, 2013). Until the pathway is active by Shh, two of the three Gli transcription factors, Gli2 and Gli3 are both phosphorylated by a PKA-dependent mechanism into their repressor form (GliR) (Pal and Mukhopadhyay, 2015). Suppressor of Fused (Sufu), a negative regulator of the Shh pathway functions to help stabilize and inhibit the activation of Gli proteins (Dessaud et al., 2008, Pal and Mukhopadhyay, 2015).

Once activated by the binding of Shh to Ptch, the inhibition of Smo is relieved, accumulates in the cilium and activates the three transcription factors of the Gli family (Gli1, Gli2, or Gli3) (Jacob and Briscoe, 2003). The induction of the signaling cascade blocks the proteolytic cleavage of Gli3, allowing it to function in its activator form (GliA) (Stamataki et al., 2005, Pal and Mukhopadhyay, 2015). These transcription factors can translocate into the nucleus and carry out their role in activating or repressing transcription of specific target genes that promote ventral domain characteristics (Dessaud et al., 2008, Pal and Mukhopadhyay, 2015).

1.3.2 BMP Signaling in Ventral Spinal Cord Cell Specification

Though there is significant evidence for the importance of Shh in the ventral patterning of the spinal cord, it seems as though this isn't the only pathway helping in determining the specified cell fate of neural progenitor cells within ventral domain (Liu and Niswander, 2005, Lupo et al., 2006).

The BMP signaling pathway, which primarily functions to organize the dorsal progenitor domains of the spinal cord, has a complementary role to that of Shh signaling in the ventral region of the spinal cord (McMahon et al., 1998, Liu and Niswander, 2005). The active repression of BMP signaling is required for the normal patterning of the ventral spinal cord (Liem et al., 2000). It is known that BMP antagonists, such as noggin, chordin, and follistatin, are secreted from the notochord and in the paraxial mesoderm surrounding the ventrolateral neural tube. In addition to the antagonizing function of Follistatin, it is also known to enhance the activation

of Shh signaling so that progenitor cells adopt a more ventral fate (Liem et al., 2000, Liu and Niswander, 2005, Lupo et al., 2006).

1.4 DORSAL SPINAL CORD CELL FATE SPECIFICATION

In the spinal cord, neural crest cells, commissural neurons and specific groups of sensory interneurons are formed dorsally (Liu and Niswander, 2005). The dorsal neural progenitor cells are subdivided, much like the ventral spinal cord progenitor domains, as shown by the expression of different bHLH and HD transcription factor genes into eight distinct dorsal domains (Chizhikov and Millen, 2005). There are six early-born post-mitotic dorsal neuron populations, denoted dI1-dI6 and two later born postmitotic populations, dILA and dILB (Helms and Johnson, 2003). Further classified by their dependence on roof plate signaling for formation: Class A (dI1-dI3) neurons are dependent on BMP/Wnt signaling, whereas class B (dI4-dI6, dILA/B) is independent of roof plate signaling (Chizhikov and Millen, 2005). A number of know downstream targets of the BMP and Wnt signaling pathways have been identified that function as cell type specific determinants in Class A progenitors.

1.4.1 BMP signaling in Dorsal Spinal Cord Cell Fate Specification

Early development, BMP is initially inhibited to allow neural fate determination of the dorsal ectoderm (Lee and Jessell, 1999). TGFβ family proteins pattern the dorsal spinal cord tissue and arise and are secreted from the overlying ectoderm and the dorsal roof plate (Liu and Niswander, 2005). These signals include BMP2, BMP4,

BMP5, BMP7, GDF7, Activin and dorsalin (Liem et al., 1995, Liu and Niswander, 2005, Wilson and Maden, 2005, Le Dreau and Marti, 2012). Dimers of BMP ligands induce the stabilization of a receptor complex containing two Type-1 and two Type-2 receptors. The type-2 receptors, (BMPR2, ActR2a, and ActR2b) activate the Type-1 receptors (BMPR1a and BMPR1b) via phosphorylation (Le Dreau and Marti, 2013). This activation of the Type-1 receptors induces phosphorylation of intracellular R-Smad factors (Smad1/5/8) (Liu and Niswander, 2005, Ulloa and Briscoe, 2007). Phosphorylation of R-Smad factors allows them to interact with Smad4, which stabilizes the Smad complex, allowing its translocation into the nucleus. In the nucleus, the Smad complex can regulate transcription of the dorsal target genes (Liu and Niswander, 2005, Le Dreau and Marti, 2012). When different variations of BMP signaling are knocked out, more specifically BMP4, the dorsal spinal cord does not develop properly.

1.4.2 Wnt/β-catenin signaling in Dorsal Spinal Cord Cell Fate Specification

Wnt1 and Wnt3a are expressed in the roof plate and surrounding region in both mouse and chick embryos (Parr et al., 1993, Hollyday et al., 1995, Megason and McMahon, 2002, Le Dreau and Marti, 2012). Wnt expression is first detected in the roof plate as soon as the neural tube closes and continues to be expressed throughout neurogenesis. It is believed that the canonical-Wnt pathway is the WNT signaling pathway involved in patterning, proliferation, and cell determination (Le Dreau and Marti, 2012).

Wnt signals through a membrane bound receptor called frizzled, which is bound to low-density lipoprotein receptor related protein 5 and low-density lipoprotein receptor related protein 6 (LPR5/LPR6) (Bhanot et al., 1996, Tamai et al., 2000, Komiya and Habas, 2008, Le Dreau and Marti, 2012). The binding of Wnt activates an intracellular cytoplasmic scaffold protein, Dishevelled (Dvl). This induces disassembly of a complex consisting of molecules Axin, Adenomatosis (APC), glycogen synthase kinase 3beta (GSK3β), and β-catenin (Ciani and Salinas, 2005, Komiya and Habas, 2008). This disassembly inhibits the phosphorylation of β-catenin by GSK3β, allowing for the increase of β-catenin levels in the cytoplasm. The build up of β-catenin levels in the cytoplasm leads to translocation into the nucleus. Once in the nucleus, β-catenin forms a complex with T-cell specific (TCF) transcription factor (Inestrosa and Varela-Nallar, 2015). (Fig: 1.2).

It has been shown when overexpressed, the downstream target of the canonical-Wnt pathway β-catenin causes an increase in dorsal and intermediate expression patterns of genes such as Pax7 and Pax6 ventrally at the expense of ventral progenitor markers, Nkx6.1, Olig2, and Nkx2.2 (Alvarez-Medina et al., 2008, Yu et al., 2008, Le Dreau and Marti, 2012). In addition, double knockout of Wnt1 and Wnt3a in mice produces fewer dI1-3 interneurons at E10.5 as shown by pro-neural genes, such as Math1 and Ngn1, and by markers in committed dorsal neurons such as Lh2 and Isl1 (Chizhikov and Millen, 2005).

It appears that Wnt/β-catenin also play a role in inhibiting Shh signaling activity in the dorsal spinal cord by targeting Gli3. It is reported that increased expression of Gli3 in its repressor form represses Shh activity. It is suggested that this activation of Gli3R expression by the Wnt signaling pathway within the dorsal neural tube is what serves to restrict Shh activity (Le Dreau and Marti, 2012).

1.5 CONVERGENT EXTENSION

During development, the spinal cord must also elongate rostrocaudally. It is known that early on in gastrulation and neurulation, convergent extension movements drive elongation of the embryo along its anterior-posterior (AP) axis. Convergent extension is known as the lengthening and narrowing of a field of cells and it contributes to a variety of morphogenetic processes (Tada and Heisenberg, 2012). It was first postulated by Vogt in 1922 that convergent extension involved active rearrangement of cells.

During convergent extension, neuroepithelial cells become polarized (Wallingford et al., 2002, Montcouquiol et al., 2006, Tada and Heisenberg, 2012). This polarity is known as planar cell polarity and is important to allow the formation of the neuroepithelial sheet to move in a coordinated manner (Montcouquiol et al., 2006, Tada and Heisenberg, 2012, Yang and Mlodzik, 2015). This directly leads to elongation of the anteroposterior axis. The hindbrain and spinal cord narrow and elongate dramatically during neurulation and like in the mesoderm, neural

convergent extension is driven by cell intercalation (Keller et al., 1992). If planar cell polarity is affected then the neural tubes fail to form. The Wnt signaling pathway

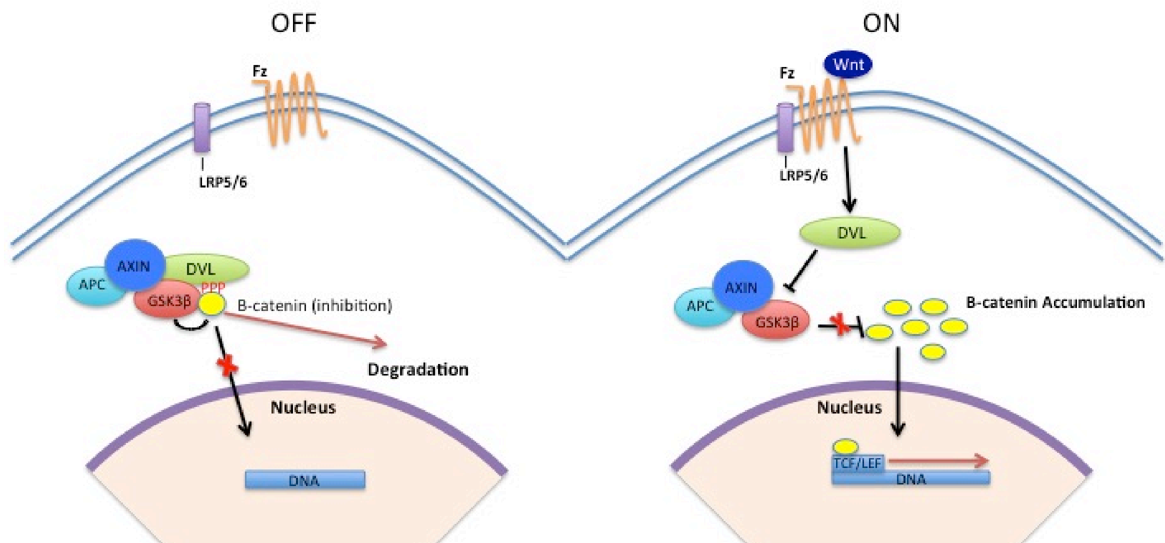

Figure 1.2: Activation of the Wnt signaling pathway in the Neural Tube: Wnt binds to Fz, which is bound to LRP5/6. This activates Dvl, which disassembles the Axin, Apc, Gsk3beta, and beta-catenin complex, inhibiting phosphorylation of beta-catenin by Gsk3beta. Increased levels of Beta-catenin in the cytoplasm allows for translocation into the nucleus, targeting the TCF transcription factor. (Figure modified from Ciani & Salinas, 2005).

plays a variety of different roles in spinal cord development, one of which is helping control the planar-cell polarity pathway (Wallingford et al., 2002).

In Drosophila, the planar cell polarity (PCP) pathway is controlled by the non-canonical-Wnt signaling pathway, which is also known as the PCP cascade. (Wallingford et al., 2002) This pathway uses Wnt components such as Frizzled and Dishevelled but does not involve other classic canonical Wnt pathway molecules like GSK-3, Axin, or β-catenin (Wallingford et al., 2002). This PCP pathway instead interacts with Strabismus (Stbm), Prickle, and JNK (Wallingford et al., 2002). Analysis of cell behaviours during convergent extension revealed a strong connection between PCP signaling and mediolateral cell polarity (Wallingford et al., 2002). A similar mechanism is at work in most vertebrate animal models. Detected within the chick and the mouse model, the extension of the notochord and neural tube involved cell rearrangement (Wallingford et al., 2002).

1.6 ANTEROPOSTERIOR PATTERNING

Signaling along the anteroposterior axis of the neural tube subdivides the CNS into four main areas: the forebrain, midbrain, hindbrain and spinal cord (Jung and Dasen, 2015). In both flies and vertebrates, it was discovered there are genes with a known patterning role expressed in spatially restricted domains of the neural plate and tube. During axis elongation, progenitor cells are exposed to graded levels of signaling molecules leading to the progressive activation of HOX genes (Deschamps and van Nes, 2005). Hox genes are known to specify positional cues along the AP

axis. In vertebrates, the Hox genes are expressed in overlapping domains along the AP axis of the early embryo (Kessel and Gruss, 1991, Deschamps and van Nes, 2005).

It's been shown that Retinoic Acid (RA) and Fibroblast Growth Factor (FGF) act as opposing signals to modulate the expression of the Hox genes along the AP axis (Dubrulle and Pourquie, 2004). Both signals play a key role in establishing the patterns of Hox4-Hox10 genes, along the anteroposterior axis of the spinal cord. FGF signaling has been shown in the chick to be essential for the maintenance of progenitor cells in the extending neural tube (Dubrulle and Pourquie, 2004). The concentration of FGF expression is gradually reduced, moving to more anterior positions and it is thought that this reduction helps to signal the transition from proliferating neural progenitors to differentiated neurons moving out of the ventricular zone (Dubrulle and Pourquie, 2004). Increasing levels of FGF can induce Hox genes with a progressively more posterior character.

Retinoic Acid, produced in somitic-mesoderm more anteriorly positioned acts to stimulate maturation and differentiation of neural progenitor cells. It is thought the integration of both signals act as a switch that coordinates the patterning of the extending spinal cord with that of the mesoderm (Lara-Ramirez et al., 2013).

What's further and more recent, the Wnt pathway is speculated to also play a role specifying the different anteroposterior positions of the neural plate and neural

tube in a dose-dependent fashion. Moving from the posterior region anteriorly along the neural plate, there are specific Wnt inhibitors secreted. A key regulatory event in the process of caudalization of the spinal cord is the inhibition of Cyp26 in the neural plate by FGF and WNT signaling, this allows for a greater concentration of RA expression (Kudoh et al., 2002, Lara-Ramirez et al., 2013, Philippidou and Dasen, 2013).

1.7 NOS1AP

First identified in 1998 by Jaffrey et al, Nitric Oxide Synthase Adaptor Protein (NOS1AP), also known as carboxy terminal PDZ ligand of nNOS, contains 2820 base pair complementary DNA that translates to a 503 amino acid protein (Jaffrey et al., 1998). NOS1AP has been shown to localize throughout the central nervous system, with the highest mRNA levels detected in the olfactory bubs, hippocampus, cortex and cerebellum (Jaffrey et al., 1998). More recently NOS1AP has been shown within the developing spinal cord (Clattenburg et al., 2015). NOS1AP contains a carboxyl-terminal PDZ binding motif and an amino-terminal phosphotyrosine-binding (PTB) domain (Jaffrey et al., 1998). The PTB and PDZ interacting motifs are essential for interactions with other proteins. Across all proteins, the PTB domain has been known for its importance in forming multiple protein complexes (Smith et al., 2006). It typically recognizes specific sequences on target proteins such as Asparagine-Proline-X-Phospho-Tyrosine or N-P-X-pY, where X can be any amino acid. The PTB domain can also recognize a non-phosphorylated N-P-X-Y motif or an N-X-X-Y motif (Smith et al., 2006).

The second major protein interaction motif that NOS1AP contains is the carboxyl-terminal PDZ binding motif. PDZ domains consist of five or six β-strands and two α-helices (Jemth and Gianni, 2007). They are known to interact with specific types of sequences found at the C-terminus of the interacting protein. PDZ domains have the ability to dimerize and form homo- and hetero-oligomers with one another. PDZ domains are also capable of binding internal sequences and may also interact with other protein binding motifs including ankyrin repeats, spectrin repeats and LIM domains (Hung and Sheng, 2002). Because of their ability to bind to specific recognition sequences often at the C-terminus of many trans-membrane proteins and have the ability to dimerize with other PDZ containing proteins, it is suggested that PDZ proteins are involved with organization at the plasma membrane in locations such as tight junctions and are often associated with scaffold proteins at those locations (Fanning and Anderson, 1996).

1.7.1 NOS1APc

Previous work in the Fawcett lab had identified an alternate isoform of NOS1AP called NOS1APc (Richier et al., 2010, Clattenburg et al., 2015). The NOS1APc isoform was first identified while performing Western blot analysis where a slower migrating band was detected at 100kDa. It was identified using bioinformatics as a longer isoform of NOS1AP known as NOS1APc. The NOS1APc isoform retains the PTB domain at the N-terminal region of NOS1AP. A 30kDa extension at the C-terminal end effectively removes the PDZ domain present in NOS1AP. When

NOS1APc was cloned from rat cerebellum, it was observed that the last exon in NOS1APa is deleted and the PMAQ sequence is spliced with VDHSMFEN of NOS1APc (Richier et al., 2010, Clattenburg et al., 2015).

1.7.2 NOS1AP interacts with Scribble, a regulator of the Hippo Pathway

NOS1AP was first identified as an associating protein with nitric oxide synthase (NOS), which gave rise to its name (Richier et al., 2010). Direct interaction between the C-terminal region of NOS1AP and the PDZ domain of nNOS was shown. Recently it has also been shown in the Fawcett lab that NOS1AP associates with the tumour suppressor protein scribble (Richier et al., 2010, Clattenburg et al., 2015). This interaction seems to be a direct interaction between the amino-terminal region containing the PTB domain and the fourth PDZ domain of Scribble. Scribble, known as a member of the LAP family of proteins, contains 16 leucine rich repeats (LRR) at the N-terminus and four C-terminal PDZ domains (Bilder and Perrimon, Bryant and Huwe, 2000). Scribble has been known to play an important role in actin dynamics, as well as the establishment of the apical-basal axis of epithelial cells through a complex with Discs-Large and lethal giant larvae (Zhao et al., 2011). A mutation in any of the three causes a loss of apical-basal polarity. Scribble functions as a tumour suppressor protein and loss of Scribble leads to the development of tumours (Zhao et al., 2011).

1.8 THE HIPPO PATHWAY

A fundamental principle of biology is the mechanism by which the size of organs is specified. As an embryo develops, cells proliferate, differentiate or undergo apoptosis. The regulation of these developmental phenomena is a delicate process carried out by multiple signaling pathways. Of these signaling pathways historically known to regulate organ size, is the Hippo Pathway (Michalopoulos and DeFrances, 1997, Lin et al., 2013).

The function of the Hippo pathway in organ size determination and tumor suppression has been confirmed in genetically engineered mouse models. It has been shown that overexpression of the downstream target of the pathway, Yes Association Protein-1 (YAP) results in enlarged livers that return to normal size post-termination of YAP expression (Michalopoulos and DeFrances, 1997). The Hippo Pathway has been known to play a role in tumour suppression. In different human cancer models, as well as in a mouse model of breast cancer there is genomic amplification of YAP. In addition to genomic amplification, it has been confirmed in various forms of human cancers that there is an elevated level of YAP protein and nuclear localization leading to the idea that alterations of YAP may have prognostic value (Lin et al., 2013).

In addition to its role in organ size determination and tumor suppression, the Hippo pathway is suspected to play a regulatory role in stem cell and progenitor cell self-renewal and expansion as well as inhibiting proliferation and promoting cell

apoptosis. YAP and TAZ have been shown to regulate embryonic stem cell self-renewal in response to TGFβ/BMP signaling (Hiemer and Varelas, 2013).

1.8.1 The Hippo Pathway in Drosophila

The Hippo pathway has emerged in the past decade and was first heavily studied in Drosophila, where it was identified as an evolutionarily conserved regulator of organ size. (Zhao et al., 2011) The first information indicating a role for the Hippo pathway in organ size control was found by genetic and proteomic screens. There were 35 identifiable Hippo pathway proteins in both fly and mammalian models (Harvey et al., 2013). These 35 proteins were grouped into three main classes, the Core Kinase Cassette, downstream transcriptional regulators and upstream branches (Irvine, 2012, Harvey et al., 2013). The first class was the Core Kinase cassette and the first components of the Hippo pathway to be identified in Drosophila using genetic mosaic screens. These components were tumour-suppressor genes warts (wts), hippo (hpo), Mod-as-tumor suppressor (Mats) and Salvador (sav). Mutation of these genes caused extreme tissue overgrowth. (Zhao et al., 2011) Studies show Hpo directly interacts with Sav to phosphorylate and activate the complex formed by Wts and another core Hippo protein Mats. (Zhao et al, 2011). The transcriptional co-activator Yorkie (Yki) was identified as a downstream effector of the Hippo pathway. This was done by showing that Wts directly phosphorylates Ser168 of Yki, a transcriptional co-activator known as a downstream growth regulatory effector (Zhao et al., 2011). This creates a binding site for 14-3-3 proteins, which promotes nuclear exclusion and cytoplasmic

accumulation of Yki. As a transcriptional co-activater it is known that Yki does not bind to DNA directly, instead, it binds DNA-binding transcription factors, such as TEAD/TEF family transcription factor Scalloped (Sd). Sd has been shown to mediate Yki-induced tissue overgrowth (Zhao et al., 2011).

There are a number of proteins that act upstream in the Hippo pathway from this main core kinase cassette. Merlin, Expanded and their interaction protein Kibra, two protocadherins Fat and Dachsous, the apical transmembrane protein Crumbs (Crb) and the CK1 family kinase Disc overgrown (Dco) all have shown to possess the ability to activate the hippo pathway (Zhao et al., 2011, Irvine, 2012).

1.8.2 Hippo pathway in Mammals

The hippo pathway is also known to function in mammals as a regulator of organ size control. Many of the core components and downstream effectors of the Drosophila hippo pathway are conserved within the mammalian model. (Zhao et al., 2011) There are two Hpo homologs (Mst1 and Mst2), one Sav homolog (Sav1), two Wts homologs (Lats1 and Lats2), and two Mats homologs (MOBKL1A and MOBKL1B, collectively referred to as Mob1). These proteins form the conserved kinase cassette that phosphorylates and inactivates the mammalian Yki homolog, YAP, and its paralogue TAZ (Zhao et al., 2011).

In mammals, the Hippo pathway is triggered in part by cell-cell contact. High cell density activates the Hippo pathway and induces YAP phosphorylation and

cytoplasmic translocation (Zhao et al., 2011). Activation of the core components Mst1/2 leads to phosphorylation and activation of Lats1/2. Mob1 is also phosphorylated by Mst1/2 and forms a complex with Lats1/2. Activated Lats1/2 phosphorylates Ser127 in YAP/TAZ, which promotes 14-3-3 binding and subsequent cytoplasmic sequestration and inactivation (Zhao et al., 2011). Lats1/2 phosphorylate another serine site, Ser381 that is thought to prime YAP for subsequent phosphorylation by CK1 delta/epsilon. This is followed by ubiquitin-mediated degradation of YAP (Zhao et al., 2011). (Fig: 1.3).

YAP/TAZ/Yki can also be inhibited through protein-protein interacts resulting in cytoplasmic sequestration. Recently, alternative ways to sequester YAP to the cytoplasm have been identified. For example, YAP/TAZ and the Angiomotin family proteins interact causing tight junction localization of YAP/TAZ and inhibition through phosphorylation dependent and –independent mechanisms (Zhao et al., 2011).

There are multiple known downstream transcription factors that can partner with YAP to enable DNA-binding. Such transcription factors are p53 family member p73, the Runt family member Runx2, Smad1 and the TEAD/TEF family member transcription factors (Zhao et al., 2011). Of all of these, it appears the TEAD/TEF family transcription factors, which are homologous with Sd in Drosophila, are the prime mediators of YAP/TAZ in the Hippo signaling pathway (Zhao et al., 2011, Harvey et al., 2013) (Fig: 1.3). The knockdown of TEAD abolishes YAP dependent

gene transcription and largely diminishes YAP-induced cell proliferation, oncogenic transformation and the epithelial-to-mesenchymal transition (EMT) (Zhao et al., 2011).

Upstream of the core kinase cassette in mammals, a number of hippo pathway regulators have been identified. Consistent with the core kinase cassette proteins and downstream targets, homologues of the upstream regulators found in the Drosophila have been reported in the mammalian model. These upstream regulators identified to date include two Kibra (Kbr) homologs (KIBRA/WWC1 and WWC2), two possible Expanded homologs (FRMD6/Ex1 and FRMD1/Ex2), one Mer homolog (NF2/Mer), one Ft homolog (Fat4/Fat-j), two Ds homologs (Dchs1 and Dchs2), one Fj homolog (Fjx1), two Lft homologs (Lix1 and Lix1-like), and three Crb homologs (Crb1, Crb2, and Crb3).

The Hippo pathway has been implicated to function in the development of many different organelle systems. More recently the hippo pathway is speculated to play a role during neurogenesis (Cao et al., 2008). The overexpression of YAP within the neural tube of the developing avian embryo leads to an increase the in the neural progenitor pool, coupled with loss of differentiated cells as reported by the Pfaff lab in 2010.

1.8.3 YAP

Yes-association protein 65 (YAP), a downstream target of the Hippo signaling pathway, functions as a transcriptional co-activator and as a scaffolding protein (Gee et al., 2011). YAP is known to be a founding member of the WW domain-containing protein family, having two WW domains (Gee et al., 2011). This WW domain allows the binding of proteins containing a PPxY motif such as P53 family members Smad7, Runx2, Pax and ErbB4 (Gee et al., 2011). Aside from its WW domain, YAP has multiple protein-protein interaction domains. At its N-terminal YAP can bind to TEA domain-containing transcription factor family (TEAD/TEF), and LATS (Large tumor suppressor), while at its C-terminus YAP contains a PDZ-binding motif that allows for binding to PDZ domain-containing proteins (Gee et al., 2011).

Being the downstream co-activator of the hippo pathway YAP is known to be important for the maintenance of cell proliferation and differentiation, and instrumental in organ growth and regeneration. When YAP is deregulated it is common to observe uncontrolled proliferation, which coincides with the Hippo Pathway being a prevalent pathway in a number of different types of cancer (Piccolo et al., 2014, Plouffe et al., 2015).

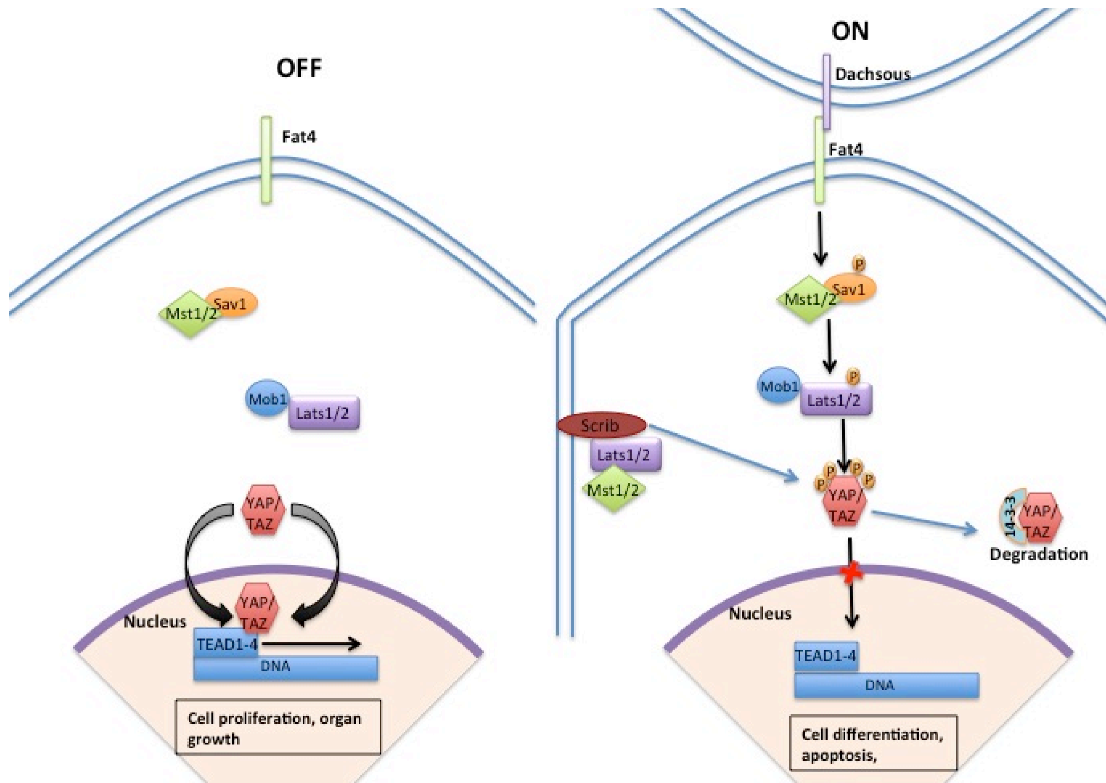

Figure 1.3: Hippo Signaling pathway in the Neural Tube: High cell density triggers activation, which activates MST1/2. MST1/2 phosphorylates Lats1/2, which then induces phosphorylation of YAP/TAZ. Phosphorylation of YAP/TAZ sequesters YAP/TAZ to the cytoplasm for degradation. Scrib also acts through Lats1/2 to inhibit YAP/TAZ translocation into the nucleus. (Figure modified from Harvey et al. 2013).

1.8.4 Yap plays a regulatory role in Neurogenesis

As stated before, it is known that tight control of cell proliferation is essential for proper growth during development. Though there has been an immense amount of research done surrounding the hippo pathway and it's function in organ size regulation, it's only been within the last decade that the potential role in regulation the hippo pathway could have on neural progenitor cells undergoing proliferation has been explored. Reported by experimental studies done by Pfaff et al. in 2008, manipulating the hippo pathway, more importantly, the expression of YAP, disrupted neurogenesis in the developing neural tube of avian embryos. To study the function of the Hippo pathway in vertebrate neural development, they began by first examining the expression pattern of YAP in the chick neural tube (Cao et al., 2008). Observing the expression at HH stage 27-28, YAP mRNA was detected in the ventricular zone. Using an antibody specific for phosphorylated YAP, area of expression for YAP matched that of the ventricular progenitor zone, demarcated by the neural progenitor cell marker Sox2. This expression pattern of pYAP suggested that the Hippo signaling pathway was activated in neural progenitor cells (Cao et al., 2008).

In order to explore this idea, a constitutive YAP expression clone was electroporated into one side of the chick neural tube at HH12-14. At this point in development, it is known that the neural tube is composed of almost entirely progenitor cells (Cao et al., 2008). It was shown on the transfected side this overexpression of YAP caused a decrease in neuronal differentiation, at 22 hours post-electroporation and at 45

hours post-electroporation there was a marked increase in Sox2+ progenitor cells (Cao et al., 2008). The increase in progenitor cell number became more prominent 3 days post-electroporation. The transfected side was significantly broader than the control side. It was noted that ectopic Sox2+ cells in the mantle zone continued to proliferate through 5-bromodeoxyuridine (BrdU) labeling. There was also the presence of ectopic phospho-histone H3+ mitotic cells around foci of aPKC+ adherens junctions. As these Sox2+ cells were contained in circular clusters, this indicated that the overexpression of YAP induced the formation of Sox2+ positive rosettes (Cao et al., 2008).

Although there is speculation as to which of the transcription factors/cofactors interact with YAP to regulated cell proliferation in the hippo pathway, it has been supported by evidence-based studies that TEAD participates in YAP-mediated gene regulation in neural progenitor cells. It is known that there are at least three TEAD proteins, TEAD1, TEAD3 and TEAD4 encoded in the chick genome (Cao et al., 2008). It was shown using an antibody specific for TEAD1 that at the cervical, brachial and lumbar levels it was expressed in the ventricular zone, but not the Tuj1+ mantle zone. A closer look showed that the TEAD1 region was slightly broader than that of pYAP and Sox2+ expression (Cao et al., 2008). TEAD4 mRNA was also expressed in the ventricular zone of the chick neural tube. Expressing a transcriptionally active form of TEAD1 into the neural tube produced phenotypes that were similar to those produced by YAP overexpression. There was a decrease in neuronal differentiation

at 22hours post electroporation, expansion in neural progenitor pool at 45 hours post electroporation and by 69 hours post electroporation there tumor-like rosette formations in the ectopic region. Despite the structural distortion of YAP and TEA-VP61-transfected neural tubes, dorsal-ventral patterning determined regional markers Pax3, Pax7, and PAx6 was largely normal (Cao et al., 2008).

To verify whether or not YAP caused these phenotypes by exerting its effects through TEAD 30 amino acids were removed from the TEAD-binding domain. Confirmed by a co-immunoprecipitation experiment, this mutant no longer interacted with TEAD1. When this mutant form of YAP was overexpressed in the neural tube it did not significantly increase neural progenitor cells (Cao et al., 2008).

1.9 CHICK EMBRYO MODEL – *In Ovo* electroporation

As it is known, animal models play a critical role in elucidating different pathways known to function in development. The chick embryo has provided crucial insight into the development and organogenesis, and has shown to be a useful model for studying embryonic stages and embryo maturation ex utero until hatching. A well characterized embryonic model, they have been widely utilized to unveil mechanisms of embryonic development and to analyze expression patterns of candidate genes at specific stages of organogenesis (Croteau and Kania, 2011).

In ovo electroporation is a technique involving living embryonic chicks. This technique allows for direct genetic modification and manipulation of a single or multiple genes depending on intended outcome. Using electric field pulses, the plasma membrane stability is disrupted transiently, creating pores in the cellular membrane, which the DNA plasmid is driven through towards the positive electrode, due to its negative charge. DNA is injected into the neural tube of the developing embryo, and almost instantaneously following the injection, a high voltage at a short duration, pulsed 3-5 times, is applied by L-shaped electrodes to the neural tube. The embryo is then placed back into the incubator for a specified duration of time. Ultimately, if the technique was successful, after the specified duration of incubation time, the embryo should express the injected DNA on one side of the spinal cord.

1.11 SUMMARY AND RATIONALE

It is evident there are multiple pathways critical in the organization and function of the developing spinal cord. Previously, the Fawcett lab has shown an interaction between Scribble and a Nitric Oxide Synthase Adaptor Protein (NOS1AP), and that this interaction is important for synapse development in the brain (Richier et al., 2010). Interestingly, evidence suggests that Scribble functions through the hippo-signaling pathway. The overexpression of YAP, the downstream target of the hippo pathway, has an effect on the differentiation of neural progenitor cells. My work has tested whether NOS1AP functions in the hippo-signaling pathway to regulate

neuronal differentiation. To investigate our theory, NOS1AP was overexpressed in the spinal cord of developing chick embryos through in ovo electroporation.

1.11 HYPOTHESIS

I hypothesize that NOS1AP plays a role in Hippo signaling and contributes to spinal cord development.

1.12 AIMS

1. **Determine whether there is an interaction between YAP and NOS1AP.**
2. **Develop the *in ovo* electroporation technique for NOS1AP injection into the spinal cord**
3. **Overexpress NOS1AP into the avian embryo spinal cord and determine whether overexpression effects neural progenitor differentiation.**

CHAPTER 2 MATERIALS AND METHODS

2.1 Antibodies

Antibodies used in this body of work include: pan-NOS1AP – a rabbit polyclonal antibody (Richier et al., 2010) used at 1:200 dilution for IP, 1:1000 for Western Blot; a NOS1APc specific rabbit polyclonal peptide antibody (pep-NOS1AP) (Richier et al., 2010), used at 1:200 dilution for IP, 1:1000 for Western blot; Pre-Immune NOS1APc – rabbit polyclonal antibody used at 1:200 dilution for IP; CAPON R-300 rabbit polyclonal antibody from Santa Cruz (Cat. No. sc-9138) used at 1:2000 dilution for immunocytochemistry; a Nestin mouse monoclonal antibody obtained from Santa Cruz (10c2) used at 1:200 dilution for immunocytochemistry; a Yap mouse monoclonal antibody was obtained from Santa Cruz (Cat. No. sc-101199) used at 1:200 dilution for IP, 1:500 for Western blot; a GFP rabbit polyclonal antibody from Abcam (Cat. No. Ab290) used at 1:2500 dilution for immunocytochemistry, 1:1000 for immunocytochemistry in the chick spinal cord; a Flag M2 mouse monoclonal antibody from Sigma 37 (Cat. No. F3165) used at 1:2500 for immunocytochemistry; a Sox2 mouse monoclonal antibody from Cell Signaling (Cat. No. 4900S) used at 1:500 dilution for immunocytochemistry in the chick spinal cord; Blotting Grade Affinity Purified Protein A-Horseradish Peroxidase Conjugate – secondary antibody from Bio-Rad (Cat. No. 170-6552) used at a dilution of 1:10,000 for Western blot; Blotting Grade Affinity Purified Goat Anti-Mouse IgG (H+L) Horseradish Peroxidase Conjugate – secondary antibody from Bio-Rad (Cat. No. 170-6516) used at a dilution of 1:10,000 for Western blot; Blotting Grade Affinity Purified Goat Anti-Rabbit IgG (H+L) Horseradish Peroxidase Conjugate – secondary antibody from Bio-Rad used

at a dilution of 1:10,000 for Western blot; bisBenzimide H (Hoescht – Sigma Aldrich – Cat. No.B2883) used at a dilution of 1:10,000 for immunocytochemistry.

2.2 Constructs

The full length YFP-NOS1AP was created as described in Richier, et al. 2010. The YFP-NOS1APc was created as outlined in Clattenburg, et al. 2015. The YFP-NOS1AP-PTB construct was described in Clattenburg et al., 2015. The YFP-NOS1AP-ΔPTB was described in Richier et al, 2010 and Clattenburg, et al. 2015. The NOS1APc RNA probe for *in situ* hybridization was used as outlined in Michael O'Brien, Master's thesis, Department of Pharmacology, Dalhousie University, 2011.

2.3 Cell Culture and Transfection

Human Embryonic Kidney (HEK) 293T cells were grown at 37^0C, 5% carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent; Cat. No.319-005-CL). Cells were supplemented with 10% heat inactivated fetal bovine serum (FBS) (Wisent; Cat. No.080450), 2mM L-glutamine (Wisent; Cat. No.609-065-EL), 100 U/ml penicillin and 100 μg/ml streptomycin (Wisent; Cat. No.450-201-EL).

For transfection of HEK 293T cells, the cells were plated onto either 3.5cm or 10cm well plates. Cells were allowed to reach a confluence between 50-70%. Cells contained in the 10cm plates were transfected by combining 750μl of serum free media (Wisent; Cat.No.319-005-CL) with 5-10μg of cDNA and 25μl of polyethylenimine (PEI; 2mg/ml). For 3.5cm plates containing HEK293T cells, 24μl

of PEI (2mg/ml) was used instead of 25µl. The transfected cell sample was vortexed and the solution was left to sit for five minutes, prior to a second vortex. The solution was then added to the cells in a drop wise manner contained in a media containing serum (Ehrhardt et al., 2006).

For transfection of COS7 cells, the cells were plated in a similar manner. For 3.5cm plates, cells were transfected by combining 250µl of serum free media (Wisent; Cat.No.319-005-CL) with 4µg of cDNA and combining 250µl of serum free media (SFM) with 30µl of lipofectamine 2000. Both combinations were vortexed and sat at RT for five minutes. The cDNA and lipofectamine 2000 mixtures were then mixed together and allowed to sit for 20-25 minutes at RT. The DMEM was then removed from the COS7 cells, and warm SFM was added. The cDNA/lipofectamine mixture was then added to cells in a drop wise manner, and left overnight at 37^0C. The SFM/cDNA/lipofectamine mixture was then removed and changed to DMEM supplemented with 10% heat inactivated FBS, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and left overnight at 37^0C.

2.4 Immunoprecipitation

The transfected cells were left to incubate post transfection at 37^0C for twenty-four to forty-eight hours prior to lysis. 48 hours post transfection cells were washed two times with phosphate buffer saline (PBS) at room temperature, then lysed in 1ml of NP40 lysis buffer (10% glycerol, 1% NP40, 20mM tris pH8.0, 37.5mM NaCl) containing 1mM phenylmethylsulphonyl fluoride (PMSF), 10µg/ml aprotinin,

10μg/ml leupeptin, and a pinch of sodium pervanadate. Cells were then left to rock for 20 minutes at 4^0C. Cells were then scraped off the plate into an eppendorf tube. Lysed cells were then centrifuged for 20-30 minutes at 13,000 rpm at 4^0C. The supernatant of the centrifuged lysed cells was transferred to a new eppendorf tube. 30μl of the supernant was taken and combined with 30μl of 2x Sample Buffer (2xSB) consisting of 250mM Tris pH 6.8, 4% sodium dodecylsulphate (SDS), 20% glycerol, 0.01% bromophenol blue and 0.4 M Dithiothreitol (DTT) to create a whole cell lysate sample. The remaining supernatant was combined with primary antibody (as indicated) and rocked at 4^0C overnight. Following overnight incubations 100μl of 10% protein A sepharose beads (GE Healthcare; Cat# 17-0780-01) was added to each sample and incubated for 1 hour at 4^0C. Samples were then washed by spinning samples for 1 minute at 3000-5000rpm. The supernatant was removed and 1ml of fresh NP40 lysis buffer was added and beads were re-suspended. This washing process was repeated three to four times. After the final wash care was taken to remove all the remaining supernatant and re-suspend the beads in 30μl of a 2X sample buffer solution containing 4%SDS, 20% glycerol, 10%2-mercaptoethanol, 0.005% bromphenol blue and 0.125M TrisHCl pH 6.8. The samples were stored at -20^0C until required for western blotting.

2.5 Western Blotting

Unless otherwise indicated all samples were electrophoresed through 10% SDS-PAGE gels. Prior to loading, samples are boiled at 95°C for five minutes. Once loaded, samples were electrophoresed using a Biorad Mini-Protein Gel apparatus in

constant voltage mode (70V through the stack, 150V through the separation gel). Following electrophoresis, proteins were then transferred to *polyvinyl difluoride membrane* (Millipore; Cat. No.ISEQ00010). Membranes containing protein were then rinsed in distilled water and then placed in blocking solution for one hour in either 5% non-fat milk in Tris-buffered saline containing 0.01% Tween (TBST) solution or 3% bovine serum albumin (BSA; Wisent, Cat. No.800-095-EG) dissolved in TBST. Following blocking for a minimum of 1 hour, antibody as indicated was added to the blocking solution and left to incubate overnight at 4°C. Following overnight incubation in primary antibody, the membranes are washed three times with TBST for ten minutes each wash, placed in appropriate secondary antibody as indicated at a 1:10,000 dilution in TBST, and washed three times in TBST for ten minutes each wash. Once washed the membrane was exposed using chemiluminescence (SuperSignal® West Pico Chemiluminescent Substrate; Thermo Scientific). Membranes were visualized post chemiluminescence using an x-ray processor.

2.6 Immunocytochemistry of Transfected Cells

Cells were grown on cover slips (18mm) using the same transfection conditions as were followed for the 3.5cm plates, as previously outlined above. The transfected cells grown on the cover slips were washed twice with 1xPBS and fixed with 4% paraformaldehyde, 4% sucrose in PBS for ten minutes at RT. Following fixation, the cells were then rinsed with PBS, three times. Cover slips were then blocked with a 4.5% BSA – 0.5% Triton- 1x PBS solution for 1hr. The cover slips were then

incubated with in primary antibody with the appropriate dilution in 1xPBS overnight at 4°C. The cover slips were rinsed three times for ten minutes each, followed by a 1 hour incubation in an appropriately labelled Alexa Fluor secondary antibody. The cover slips were then washed extensively and then incubated with BisBenzamide (Hoescht – 33258; Sigma Cat. No.B1155) diluted 1:10,000 in PBS for five minutes. Coverslips were then dipped in sterile double deionized water and mounted onto glass slides (Fisherbrand; Cat. No.12-552-4) using Fluoromount mounting medium (Sigma; Cat #F4680).

2.7 *In situ* Hybridization

2.7.1 Tissue Preparation

The spinal cord sections (20μm) from an E11.5 embryonic mouse were collected on SuperfrostPlus glass slides (Fisher; Cat. No. 12-550-15) and immediately placed in 4% paraformaldehyde (PFA) at room temperature for 1 hour. Sections were then washed three times with 1XPBS and then once in distilled water. In order to achieve acetylation, slides in 1% triethanolamine/0.25% acetic anhydride in water. The sections were then washed twice in 0.1M PBS, once in 2X SSC, and then were placed in prehybridization buffer (50% deionized formamide, 25% 20X SSC, 12% ddH2O, 10% Denhardts solution (Sigma; Cat #30915), 2% yeast RNA (Roche; Cat. No. 10 109223001), 1% salmon sperm (Sigma; Cat. No.31149) for 4 hours. All prehybridization and hybridization steps were performed in the 58°C humid chamber, with a reservoir of 5X SSC/50% formamide located on the bottom.

2.7.2 Hybridization and NOS1APc Identification

The NOS1APc RNA probes were denatured by heating, at 80°C for 10 minutes. The slides were then treated with 150μl of hybridization mixture and covered with Parafilm and left overnight. Once the Post-hybridization buffers were equilibrated to hybridization temperature (58°C) the slides were washed by each buffer for five minutes in the following order: 5X SSC, 2X SSC, 0.2X SSC, 50% formamide/0.2X SSC. After the final wash, the slides were then placed in 0.2X SSC, and then washed twice for five minutes at room temperature in 1X detection buffer. The sections were then blocked for 1 hour at room temperature in 1X blocking buffer (88% detection buffer (1M Tris-Cl/1.5M NaCl, pH 7.5), 10% blocking reagent (Roche; Cat. No.11 096 176 001), 2% sheep serum (Sigma; Cat. No. G9023), 0.1% Triton X-100 (Sigma; Cat. No. X100). After blocking, the sections were then incubated in anti-DIG antibody-alkaline phosphatase (AP) conjugate (1:1000 in 1X blocking buffer) (Roche; Cat. No. 11 093 274 910) for 1 hour at room temperature. The sections were then rinsed with 1X detection buffer and submerged in 1X detection buffer. This process was done twice, for 15 minutes each time. Following being washed, the sections were placed in equilibration buffer (5% 1M Tris buffer pH 9.5, 0.5% $MgCl_2$, 0.3% NaCl in distilled water) for 5 minutes at RT. The sections were then developed in a solution containing 0.36% nitro blue tetrazolium chloride (NBT) and 0.33% 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in equilibration buffer until desired colorimetric reaction was reached. Three subsequent washes in 10mM Tris (pH 8.0) were performed for 10 minutes each. The sections were then rinsed in distilled water and air-dried. The slides were then coverslipped by placing glass coverslips over each

hybridized section using Fluoromount media (Sigma; Cat. No.F480).

2.8 Spinal Cord Tissue Harvesting

For E11.5 mouse embryo's the full embryo was kept intact and allowed to fix in 4% PFA for one hour at room temperature. For E18.5 rat embryos, the spinal cord was isolated before fixation by removing the organs, removing the spinal vertebrae and extracting the entire spinal cord. Isolated spinal cords were fixed for one hour at room temperature in 4% PFA. Post-fixation both intact embryo and spinal cords were cryoprotected overnight in 30% sucrose in PBS at 4°C. The following day the tissue was then submerged in OCT (VWR; Cat. No.25608-930) and flash-frozen using dry-ice/ethanol bath. Transverse sections were cut (20-30μm) using the cryostat and mounted onto slides, and sections were placed on microscope slides. Sections were then processed for immunofluorescence and stored at -20°C until needed.

2.9 *In ovo* Electroporation:

Fertile white chicken eggs were incubated for 60 hrs in a humidified, 37°C incubator until they were at Hamburger and Hamilton (HH) stage 14-16 (E2.5 equivalent) (Hamburger *et al.*, 1992). The eggs were wiped down with 70% ethanol and cracked into a plastic weigh boat. Three-four drops of a neutral red (Sigma Aldrich; Cat. No. N4638) dye solution (add a pinch of neutral red to 10mL of autoclaved double distilled water) was used to help visualize the embryo. Once the dye was set, fine forceps were used to remove the vitelline membrane directly above the developing neural tube. Once the neural tube was visualized, 2-5μg/μl of DNA was mixed with

Phenol Red (Sigma Aldrich; Cat. No.P0290) at a 1:1 and was injected into the developing neural tube.

Once the membrane was removed, a hand made electrode bent to ninety degrees at each end was laid straddling the neural tube. Each end of the electrode was placed parallel to the length of the neural tube. The positive end of the electrode was lowered such that it sat beside and below the developing spinal cord and the negative end of the electrode sat beside and just above the developing spinal cord. Three to four drops of Hanks Balanced Saline Solution (ThermoFisher; Cat. No.14025092) was added onto the center of the embryo once the vitalline membrane had been removed. Following injection of DNA (5-10sec), the electroporation machine (set at Voltage: 24V, Pulse Length: 50msec, Number of Pulses: 5, and Interval between Pulses: 1sec) hooked up to the electrode was started. Electrodes were quickly removed from the yolk and immediately following electroporation, covered embryos were placed back into the incubator for 2-3days.

Once embryos had reached the appropriate developmental stage they were then examined under a fluorescent microscope to identify successful electroporation. Embryos that were fluorescent were sacrificed and fixed in 4% paraformaldehyde ($(CH_2O]n$) for 2hrs at 4°C. Following fixation, the embryo is then washed in PBS and placed in 30% sucrose (10mL of PBS, 30g of sucrose.) overnight or until the tissue has sunk. Once sunk, the head and internal organs were removed. The embryo was then frozen in OCT and sectioned. 20nm sections were collected onto glass slides

and either stained with antibody or kept in -20°C until used.

2.10 Immunohistochemistry of Spinal Cords

For all spinal cords isolated from avian, rat and mouse embryos the same protocol as follows was used. The mounted sections are washed with 1x PBS three times. Sections were blocked in a solution containing 7% Bovine Serum Albumin, 0.3% triton (Sigma Aldrich; Cat. No.9002-93-1) in PBS for 1hr. Unless otherwise stated, all incubations were done in a humidified chamber. Following block, sections were incubated at 4°C in primary antibody diluted in 4.5% BSA – 1.5% Triton- 1x PBS solution. Following overnight incubation sections were washed three times in PBS. Sections were then incubated for 1hr in secondary antibody diluted 1:1000 in 4.5% BSA – 1.5% Triton- 1x PBS solution. A final 1x PBS wash cycle is performed; 3 times, 5 minutes each wash. Immediately after the final wash in PBS, slides were coverslipped in fluoromount and left to dry for 1-2hrs.

2.11 Imaging and Post-processing

All immunocytochemistry was visualized by confocal imaging. Confocal images were acquired on the Carl Zeiss LSM710 system. All spinal cord epifluorescent images (chick, mouse, and rat) were acquired using a Zeiss Axiovert microscope equipped with Slidebook software (3i). Line scans of the confocal HEK293T cells were done using the Carl Zeiss Zen 2009 LSM710 Release Version 6.0 SP2 software.

CHAPTER 3 RESULTS

3.1 NOS1AP and YAP Interact:

The tumour suppressor protein scribble interacts with the Nitric Oxide Synthase 1 Adaptor protein (NOS1AP) (Clattenburg et al. 2015, Richier et al., 2010). The third and fourth PDZ domains of scribble are necessary to interact with the phosphotyrosine-binding domain (PTB) of NOS1AP (Richier et al., 2010). Recently Scribble has been implicated in the Hippo signaling pathway (Zhao et al., 2011, Yu and Guan, 2013), and since NOS1AP associates with scribble, I first wanted to determine if NOS1AP could associate with members of the Hippo pathway. In particular I set out to test whether YAP associated with NOS1AP. Endogenous NOS1AP was precipitated from 293T cells using a pan-NOS1AP antibody and probed for Yap. NOS1AP associating with YAP from 293T cells confirmed previous reports from our group (Clattenburg et al., 2015)(Fig 3.1).

Since NOS1AP is a highly spliced protein (Clattenburg et al., 2015), I next wanted to determine if YAP could associate specific NOS1AP isoforms, namely with extended isoforms of NOS1AP, NOS1APc, d or e (Clattenburg et al., 2015). An antibody that recognizes these isoforms, anti-PPIT (Clattenburg et al., 2015), was used to precipitate the extended isoforms from 293T cells. Similar to the pan-NOS1AP antibody, the PPIT antibody was able to precipitate YAP, suggesting that the extended isoforms of NOS1AP are able to associate with components of the Hippo Pathway (Fig 3.1).

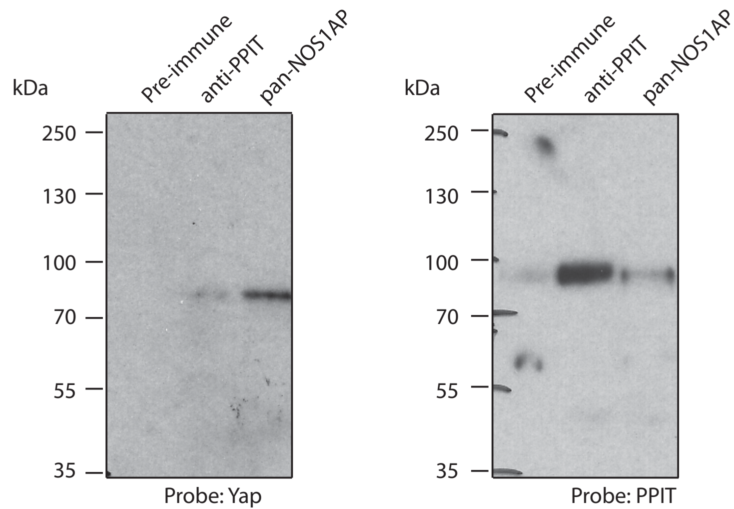

Figure 3.1 NOS1AP and YAP1 interact. NOS1APa and NOS1APc were precipitated from HEK293T cell lysate using antibodies as identified. Pre-immune serum was included as a negative control. The samples were subjected to Western blot analysis and probed with Anti-YAP antibody. Results show that NOS1APa, as well as NOS1APc (PPIT) can precipitate YAP **(Left Blot)**. To confirm whether or not NOS1APc was brought down with the pan-NOS1AP antibody, the blot was re-probed with a NOS1APc specific antibody (anti-PPIT.) NOS1APc was detected in the sample precipitated with the pan-NOS1AP antibody **(Right Blot).**

3.2 NOS1AP and YAP co-localize in HEK 293T Cells

3.2.1 YAP and NOS1AP localization

To further characterize the interaction between NOS1AP and YAP, I next transfected HEK 293 cells with cDNA's encoding YFP-NOS1APa or YFP-NOS1APc in the presence of a cDNA encoding flag-YAP. 24hours following transfection, cells were lysed and precipitated with anti-GFP antibodies and probed with anti-flag. Unlike the endogenous I.P.'s exogenously expressed NOS1AP and YAP were unable to interact, suggesting that other proteins may be necessary to mediate the interaction (data not shown).

Since overexpression of NOS1AP isoforms and YAP failed to show an interaction, I next transfected either COS7 or 293T cells with cDNA's encoding YFP-NOS1APa or YFP-NOS1APc in the presence of a cDNA encoding flag-YAP. 24hours post-transfection, cells were fixed and stained with anti-flag and anti-YFP and then imaged cells to determine the localization of YFP (i.e. NOS1AP) and flag (i.e. YAP) using confocal microscopy. YFP-NOS1APa localized at membranes and showed partial co-distributed with flag-YAP (Fig 3.2C, F). Line-scan through the cells shows the YFP-NOS1APa signal peaks with some overlap with the flag-YAP signal (Fig 3.3A,B). Although there is some overlap, the two peaks fail to completely overlap suggesting that YAP is more interior than NOS1APa consistent with NOS1APa binding membranes as has been recently reported (Clattenburg et al., 2015).

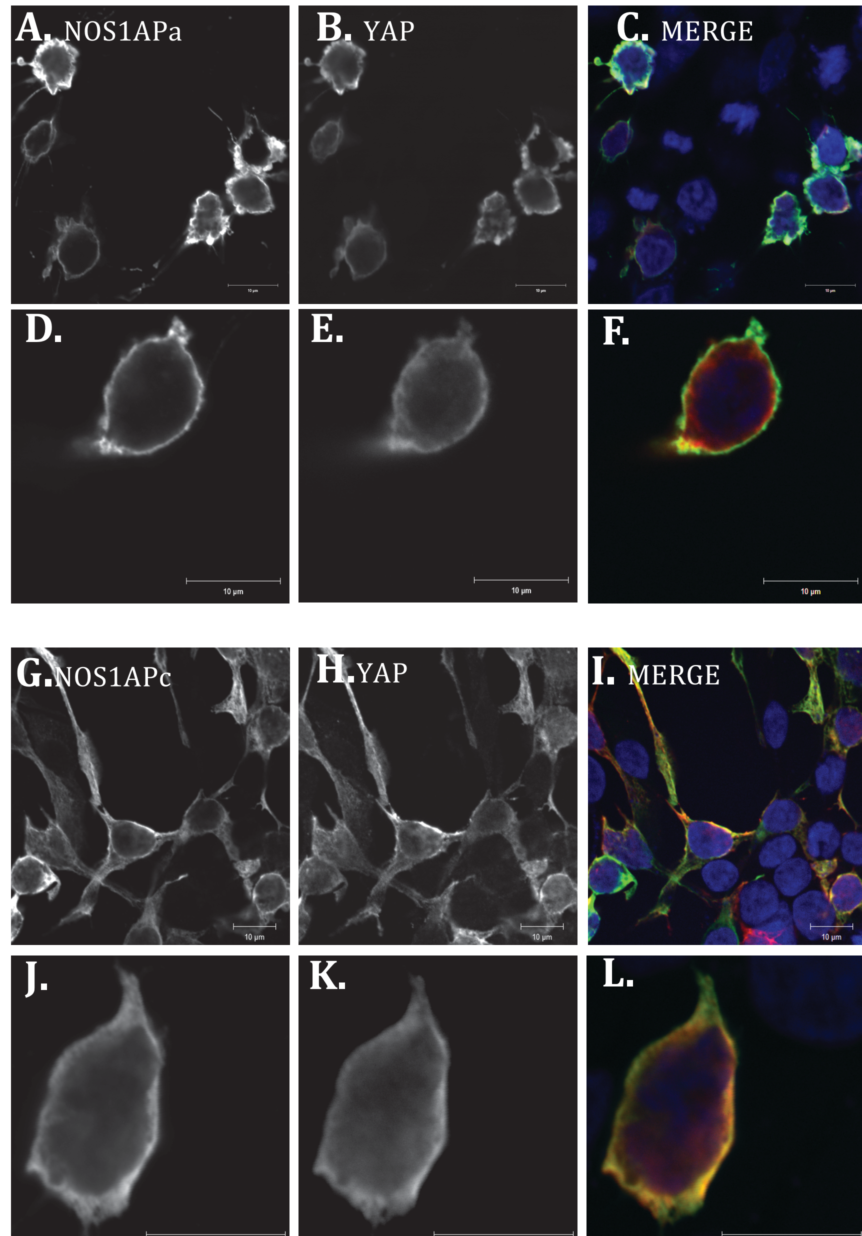

Figure 3.2A NOS1APa and NOS1APc co-distribute with YAP. NOS1APa and YAP were co-transfected in HEK 293T cells **(A-C)** Magnified single cell **(D-F)**. By performing immunohistochemistry we were able to detect an overlap in expression areas of NOS1APa and YAP in cells**(C, F)**. NOS1APc and YAP were also co-transfected in HEK 293T cells **(G-L)** Magnified single cell **(J-L)**. It appears the co-localizing expression for both NOS1AP isoforms and YAP is concentrated to the cell membrane and cytoplasm. In magnified cells, NOS1APc and YAP appear to have a more consistent co-distribution pattern at the cell membrane and cytoplasm **(L)** than that of NOS1APa and YAP **(F)**. Scale Bar = 10μm

Line scans through cells expressing YFP-NOS1APc and flag-YAP (Fig 3.3C,D) reveals an overlapping signal between the two proteins; however, unlike the localized distribution of NOS1APa, NOS1APc was more diffuse (Fig 3.2G, J). Nonetheless, there were still regions where NOS1APc and YAP did not overlap (Fig 3.2I, L). Together this suggests that exogenous YAP and NOS1AP proteins have a similar distribution in cells, with NOS1APc having a better co-distribution with YAP than NOS1APa.

3.3 YAP and the functional NOS1AP domains localization

3.3.1 Co-localization of YAP and the functional PTB domain

NOS1APa contains a PTB domain and PDZ-binding motif. To determine if specific regions of the NOS1APa protein were responsible for the association with YAP, a cDNA encoding the PTB domain fused in frame with YFP or a NOS1AP cDNA construct lacking the PTB domain (ΔPTB) fused in frame with YFP, were co-expressed with flag-YAP. YFP-PTB localized to the nucleus and membrane (Fig 3.4A, D), and showed partial co-distribution with flag-Yap (Fig 3.4C, F). Higher magnification revealed that YFP-PTB and flag-YAP co-localized in aspects of the membrane; however, there were regions where there was little co-localization (Fig 3.4C, F). Cells co-transfected with YFP- ΔPTB and flag-YAP showed co-distribution within the cell (Fig 3.4I, L), although the distribution of YFP- ΔPTB is not completely overlapping with flag-Yap (Fig 3.4L). Given that NOS1AP and YAP do not overlap exclusively may suggest that other intermediary proteins are involved in their association.

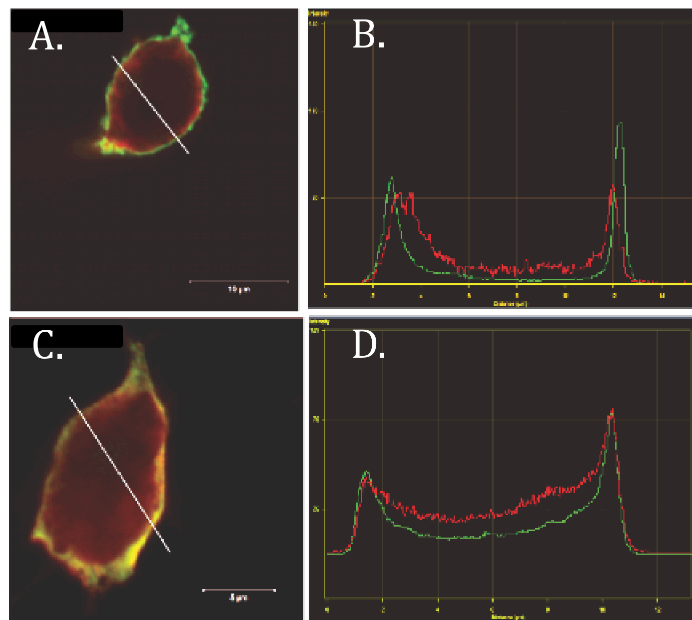

Figure 3.3 NOS1APc and YAP have a better co-distribution than NOS1APa and YAP. Line scans through cells expressing NOS1APa and YAP **(A,B)** and NOS1APc and YAP **(C,D)**. Line scans of NOS1APa and YAP show some signal overlap **(B)**, though the peaks do not overlap completely. Line scans of cells expressing NOS1APc and YAP show overlapping peak signals **(D)**, suggesting there is a better co-distribution between NOS1APc and YAP than NOS1APa and YAP.

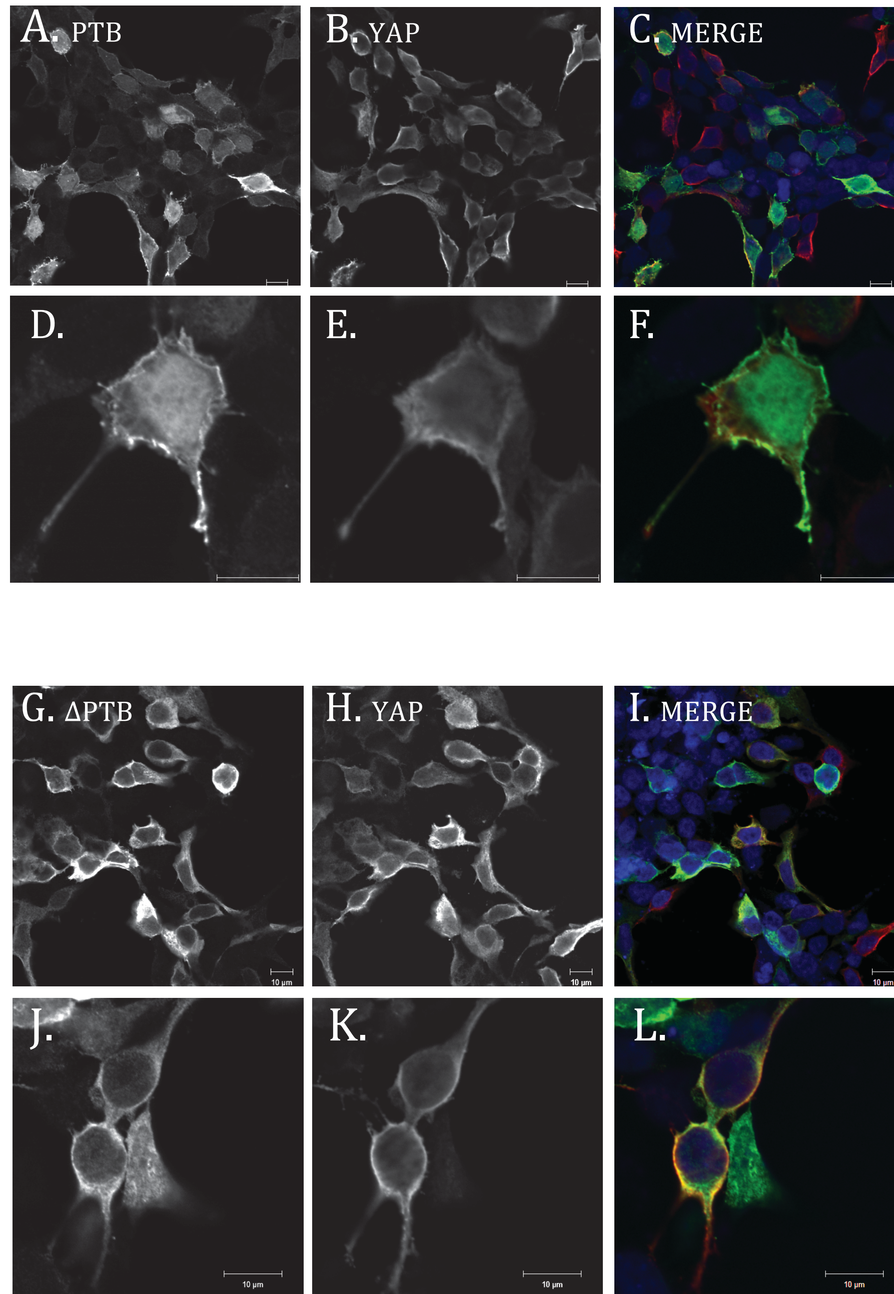

Figure 3.4 PTB and ΔPTB co-distribute with YAP.
HEK 293T cells were co-transfected with the functional NOS1AP-PTB domain and YAP **(A-F)** and NOS1AP-ΔPTB and YAP **(G-L)**. When magnifying single cells **(D-F for PTB, J-K for ΔPTB)** we were able to detect co-localization between YAP both functional domains at the cell membrane and cytoplasmic level (**F, L**). Though YAP and ΔPTB had a more consistent co-localization pattern, our data suggests either the PTB or the PDZ binding motif of NOS1AP could be responsible for the interaction with YAP. Scale Bar= 10μm

3.4 NOS1AP in the Developing Spinal Cord

Previous studies reveal that the transcriptional co-activator Yap plays a role in neurogenesis in the developing spinal cord. Since we have been able to detect an interaction between NOS1AP and Yap, we next wanted to determine whether NOS1AP was localized in the spinal cord, and whether NOS1AP played a functional role in the developing spinal cord. To date we have identified NOS1APa and NOS1APc isoforms. To determine if both are expressed in the spinal cord, we stained developing spinal cords with a pan-NOS1AP, which can detect NOS1APa, c, d, & e isoforms or an antibody that recognizes the unique region found only in the NOS1APc, d, & e isoforms. At E18.5 the pan-NOS1AP antibody revealed a limited distribution of staining that was restricted to the ventral (Fig 3.5A-B) and dorsal (not shown) midline of the spinal cord, suggesting that NOS1AP proteins are expressed in the spinal cord (Fig 3.5A-B).

Since the pan-NOS1AP antibody used to detect NOS1AP staining at E18.5 recognizes all isoforms, we next wanted to define whether the specific longer isoforms namely NOS1APc, d, e or f could be found in the developing spinal cord within the same locations. To address this, we performed an in situ hybridization using a probe that recognizes NOS1APc, d, and e isoforms (here in NOS1APc). NOS1APc mRNA was detected in the developing floor plate, roof plate and the ventral spinal cord close to the midline in E11.5 spinal cord (Fig 3.5C). In comparison to the in situ detecting NOS1APa (data not shown), there was a better distribution of NOS1APc within the developing spinal cord.

To confirm the mRNA studies, we next stained E11.5 mouse spinal cords with the PPIT antibody that recognizes the extended isoforms of NOS1AP. Consistent with the mRNA studies, highest staining was found in the roof plate, floor plate and ventral midline of the spinal cord (Fig 3.5D-F). Higher magnification revealed membrane localization and cell extensions that reached from the marginal zone to the pial surface (Fig 3.5G). This staining is similar to radial glial cells found in the developing spinal cord. Further, NOS1APc was localized to what appears to be the membranes of dividing cells, suggesting that NOS1APc is expressed in progenitor cells (Fig 3.5H-J). Together, this suggests that NOS1AP proteins are expressed in the developing spinal cord.

3.5 NOS1AP overexpression causes rosette formations in neural tube of the developing avian embryo.

The extended NOS1AP isoforms (NOS1APc, d, e) associate with Yap1, and can be found in proliferating cells within the developing spinal cord, and Yap has been shown to affect the proliferation of progenitor cells in the spinal cord. We next wanted to determine if overexpression of the extended NOS1AP isoforms could affect the proliferation of cells in the spinal cord. For this we turned to an *in ovo* system and unilaterally electroporated cDNA constructs encoding either YFP or a fusion protein encoding YFP-NOS1APc, one of the extended NOS1AP isoforms found in the developing chick spinal cord.

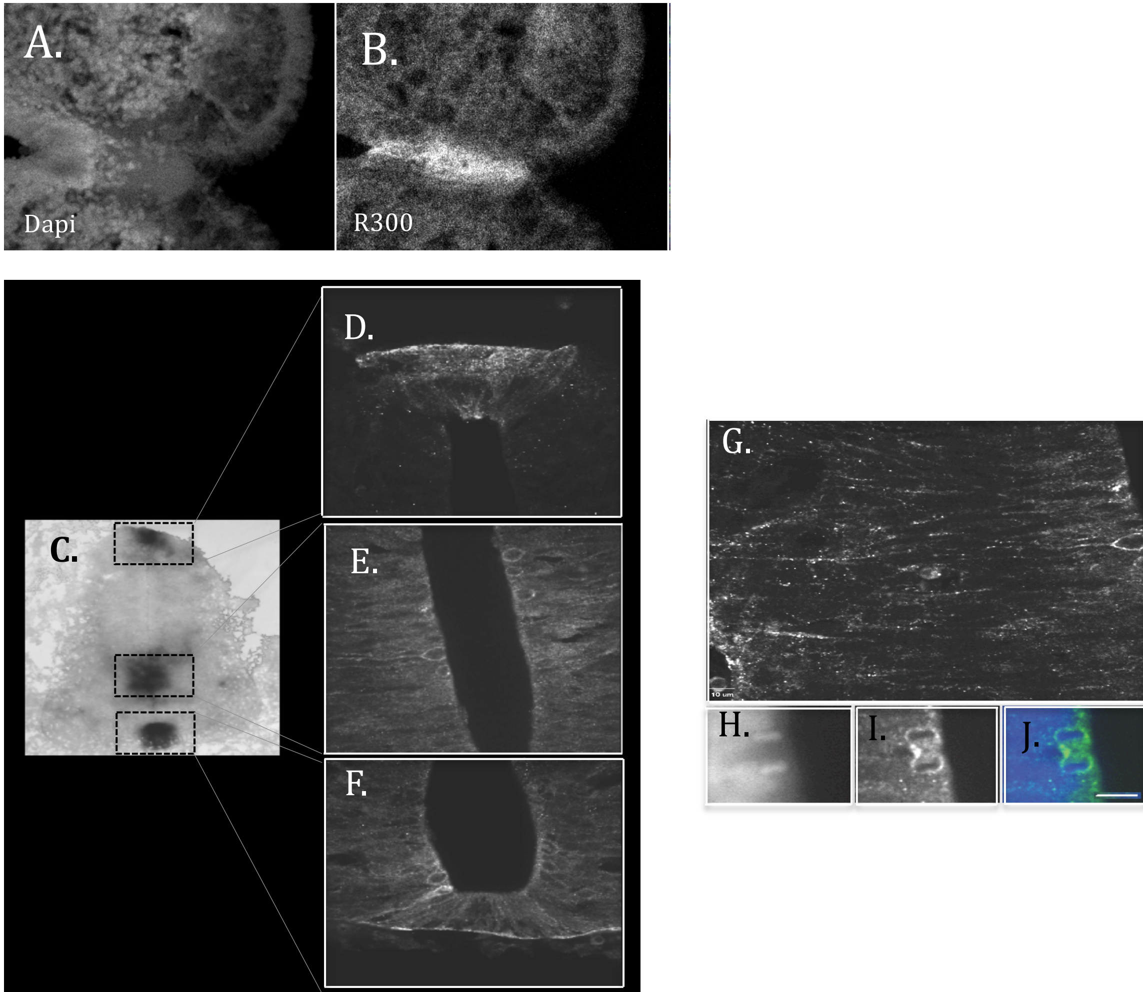

Figure 3.5 NOS1AP in the Developing Spinal Cord. (A-B)NOS1AP (R300) was detected in the developing spinal cord of an embryonic rat E18. You see there is high expression of NOS1AP in along the midline, in the proliferative ventricular zone. **(C)** NOS1APc was detected in the developing spinal of an embryonic mouse E11.5. Levels of NOS1APc mRNA were high in the roof plate, floor plate, and mid proliferative ventricular zone. **D), E),** and **F)** show NOS1APc expression by IHC. At a higher magnitude you can see NOS1APc in the, **D.** the roof plate, **E.** mid proliferative ventricular zone, and **F.** the floor plate. **(G)** There is a high expression of NOS1APc in proliferating cells in the ventricular zone, but also differentiated cells lying outside of the ventricular zone. **(H-J)** NOS1APc is found at the cellular membrane of dividing cells. **H**=Hoechst, **I**=NOS1APc, **J**=Merge

In the control spinal cords, YFP expression was found unilaterally along the dorsal ventral axis of the spinal cord, confirming our electroporation technique. A number of cell bodies and processes expressed YFP. Similar electroporation of YFP-NOS1APc lead to the expression of NOS1APc in spinal cord neurons. YFP signal was in both cell bodies and in processes extending from the ventricular surface to the pia (Fig 3.6). Interestingly, the YFP signal was detected in both the marginal zone and proliferating zone, while the YFP-NOS1APc was more restricted to the marginal zone, with few YFP positive cells found in the proliferating zone where the majority of Sox2+ve cells were found (Fig 3.6D-I)

Since Yap was shown to affect the proliferation of spinal cord cells, we tested whether the expression of YPF or YFP-NOS1APc affected the proliferation of spinal cells. To do this we monitored the expression of Sox2, a marker found in proliferative cells. In spinal cords electroporated with YFP, Sox2^{+ve} cells were localized in the mid-zone where spinal cord cells proliferate (Fig 3.6B). No ectopic expression of Sox2 expression was detected in the chick spinal cord expressing YFP alone. However, in embryos electroporated with a cDNA encoding YFP-NOS1APc, although a majority of Sox2 staining was in the proliferating midzone, the expression of NOS1APc lead to ectopic Sox2^{+ve} rosettes forming outside the marginal zone where the majority of Sox2 positive cells are known to reside. We note that there was no ectopic Sox2 expression on the control half of the spinal cords electroporated with YFP-NOS1APc (Fig 3.6E, H).

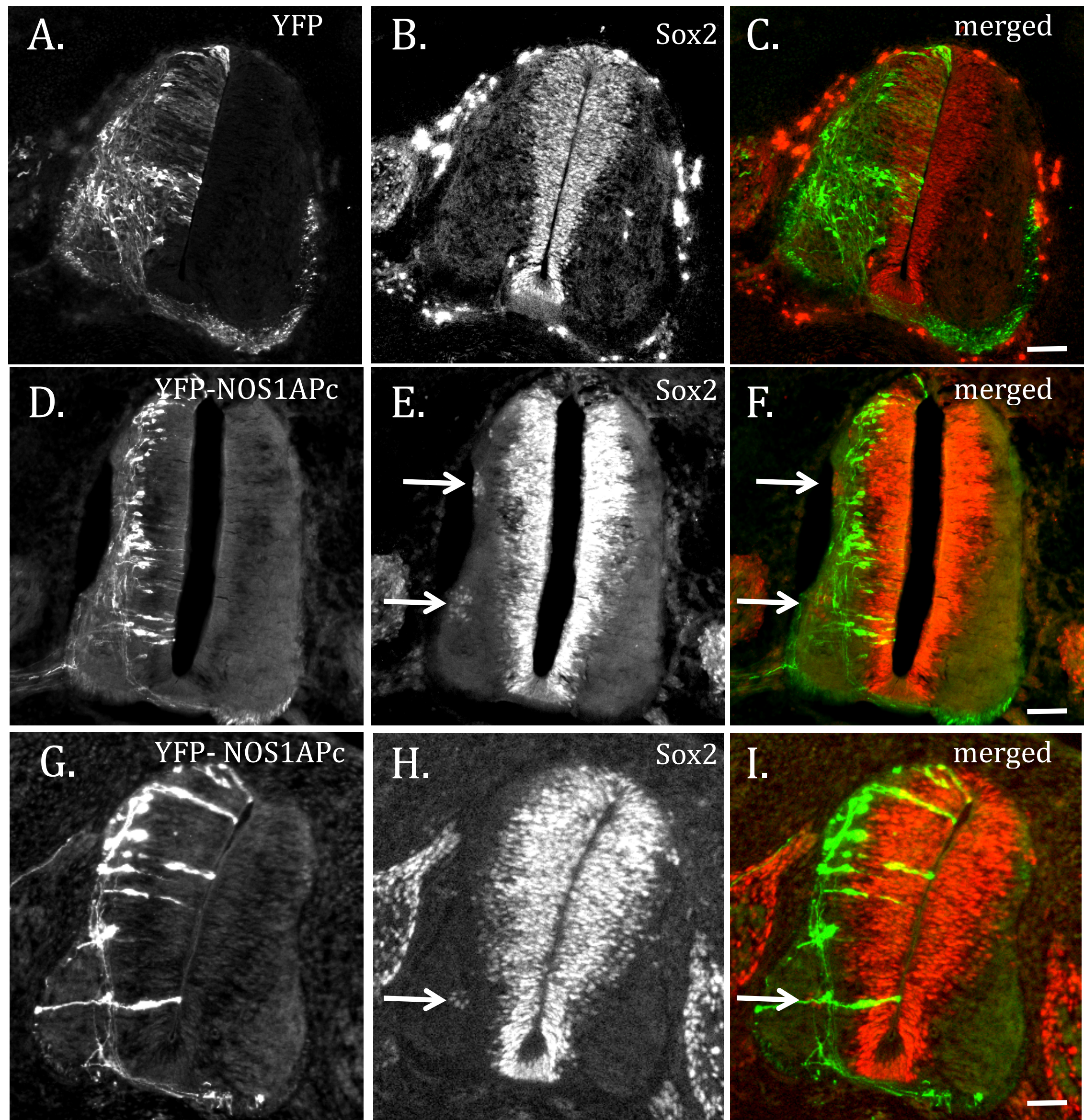

Figure 3.6 NOS1APc induces Sox2+ve Rosettes. **(Top panel)** YFP-Vector **(A)** is expressed in one half of an electroporated embryonic chick spinal chord. **(B)** Sox2 expression is observed to be unaffected, showing normal ventricular zone expression on both the control and experimental side. **(Middle Panel)** Overexpression of YFP-NOS1APc **(D)** is detected in the developing spinal cord of an embryonic chick, 2.5d.p.e. **(E, arrows)** Here you see deregulated Sox2+ expression by formation of multiple Sox2+ expressing ectopic rosette formations. **(F, arrows)** In the region where NOS1APc is overexpressed, you are able to detect the SOX2+ expressing rosette formations. **(Bottom panel)** Overexpression of YFP-NOS1APc **(G)** is detected to a lesser extent in the developing spinal cord of an embryonic chick, 2.d.p.e. One small ectopic rosettes forms in the electroporated side as reveled by SOX2+ expression **(H, arrow)**. In the region where NOS1APc is overexpressed, you are able to detect the SOX2+ expressing rosette formation **(I, arrow)**.

3.6 NOS1APc and SOX2 expression overlap

To analyze an embryo that had been successfully electroporated 2.5 days post electroporation (d.p.e), immunohistochemistry was performed using antibodies specific to YFP and SOX2. We saw that the overexpression of NOS1APc at 2.5 d.p.e resulted in ectopic SOX2 rosette formations (as stated before) only on the side of the neural tube that had been electroporated (Fig 3.7 A-B). When you magnify the areas of $SOX2^{+ve}$ rosette formations, you are able to see that there is an overlap of fluorescent cells expressing YFP-NOS1APc and the rosette formations (Fig. 3.7H, H*-I*). We detected expression of both NOS1APc positive and SOX2+ cells in the same area of the spinal cord. We noticed that when you magnify the region of SOX2 expressing rosettes, it appears as though NOS1APc and SOX2 are expressed together in a few cells, but in other cells they do not appear to co-localize. This suggests NOS1APc overexpression could be acting extrinsically or intrinsically to cause the deregulation of progenitor cells expressing SOX2 in the rosette formations.

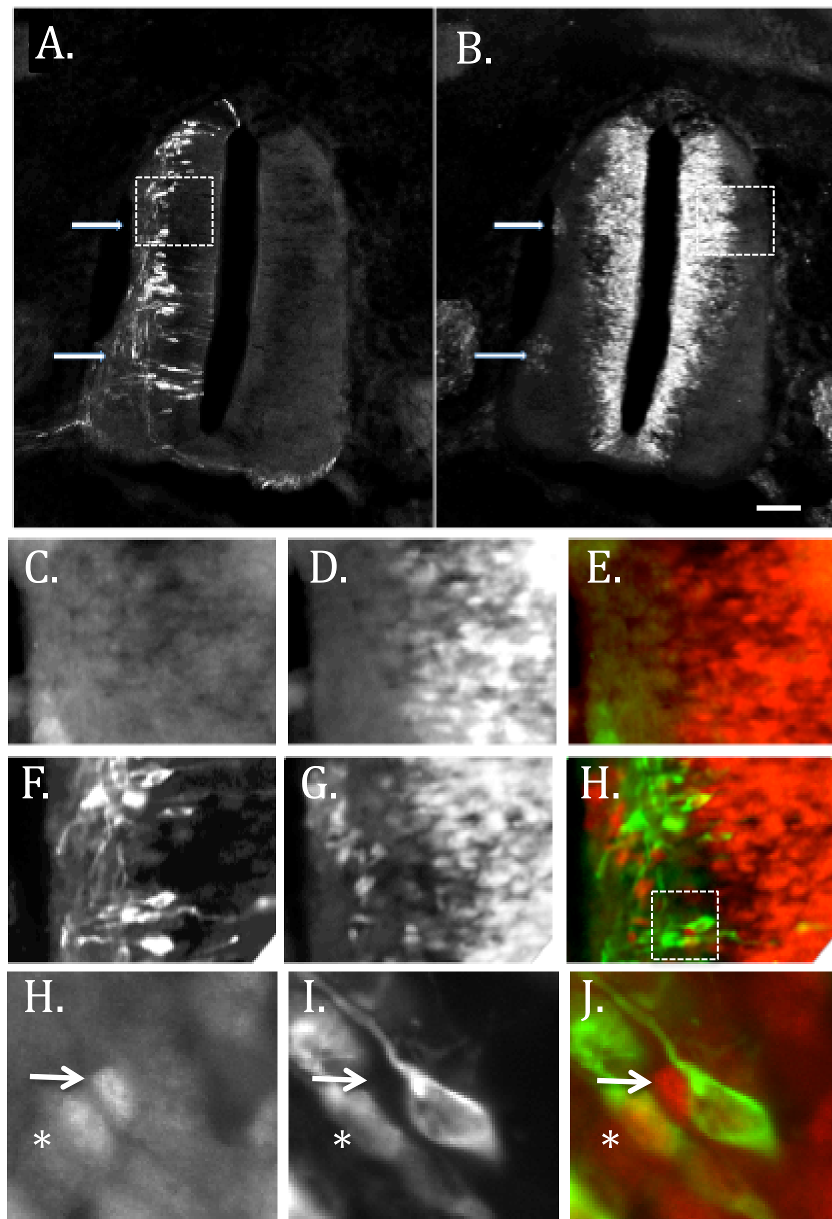

Figure 3.6 NOS1APc expression and SOX2+ Rosette formations overlap. (A) YFP-NOS1APc expression is in the same locations as the SOX2+ rosette formations (See arrows). At higher magnitude comparing the YFP-NOS1APc expressing side (**F-H**) to the control side (**C-E**) of the spinal cord you see SOX2+ expression is deregulated in the side expressing YFP-NOS1APc of the spinal cord but SOX2+ expression is normal on the control side of the spinal cord. At higher magnitude NOS1APc and Sox2+ expression are co-distributed, though it is unclear as to whether or not expression of YFP-NOS1APc and Sox2 expression directly co-localize in the same cells **(H-J*)**.

3.7 Efficacy of NOS1APc construct vs. Control Construct

Although we found the expression of NOS1AP lead to Sox2^{+ve} rosette formation, we noted that a large number of the animals were electroporated with the NOS1APc cDNA did not survive the procedure. Previous studies have shown that *in ovo* electroporation at E2.5 lead to the survival of 50-80% of the chick eggs electroporated. In my study of the 21 embryos electroporated with the YFP vector, seven survived 2 days post electroporation, and of those seven three had detectable fluorescence in the spinal cord. Thus my success rate for overall survival was 33% with a 42% success rate of having positive expression of those that survived. In comparison, a total of 235 embryos were injected with the construct encoding YFP-NOS1APc. Of these 139 or 59% survived 2 days post electroporation. Of these 139 only 3 embryos (2%) showed a strong fluorescent signal in the spinal cord, suggesting that my technique was inconsistent. (Fig. 3.8A-C)

Total Eggs Injected with YFP-NOS1APc	*Total # Embryo that lived 2d.p.e.*	*Total # of embryos that lived 2.5d.p.e*	*Total # of embryos that lived 3d.p.e*	*Total # of embryos fluorescing when sacrificed*
235	139	8	8	3
Total Eggs Injected with YFP-Empty Vector	*Total # Embryo that lived 2d.p.e.*	*Total # of embryos that lived 2.5d.p.e*	*Total # of embryos that lived 3d.p.e*	*Total # of embryos fluorescing when sacrificed*
21	7	3	3	3

Figure 3.8A This table shows the number of embryos recorded to have been injected with either YFP-NOS1APc or YFP-Empty Vector, how many lived, for how long, and how many fluoresced. This table indicates in comparison to YFP-Empty vector, embryos injected with YFP-NOS1APc had an increased mortality rate.

	Efficacy of electroporation 2 d.p.e Survival
Literature	50-80%
Control	~42%
NOS1APc	~2%

Figure 3.8B This table shows the efficacy of the construct two days post electroporation. You can see that when using our control construct, 42% of the embryos living expressed the construct at 2d.p.e. 2% of the total number of embryos living at 2d.p.e expressed the NOS1APc construct. The literature claims with a functioning construct you should have 50-80% of the embryos expressing the construct at 2d.p.e

Stage of Injection	**DNA**	**Days Post Electroporation**	**Stage**
16	YFP-Nc	2.5	26-27
16	YFP-N c	2	24-25
16	YFP-Nc	2	24-25
14	YFP	3	27-28
14	YFP	3	27-28

Figure 3.8C This table shows the number of successful animals completed to date, DNA used, days they survived post electroporation and the stage (HH) at which they were sacrificed.

Chapter 4 Discussion

4.1 Summary of Findings

My project set out to better define the role of NOS1AP and it's subsequent isoforms in the developing spinal cord, as well as to better define its potential role in regulating the Hippo Pathway. Previously, the Fawcett lab had shown that Scribble and NOS1AP interact and regulate dendritic spine development (Clattenburg et al., 2015, Richards et al, 2010). Scribble, a large scaffolding polarity protein, is reported in both genetic and biochemical studies to play a role in regulating the Hippo pathway. However the mechanism for the control of Hippo pathway signaling by Scribble still remains unknown. (Richier et al., 2010) I first sought out to determine whether there was an interaction between NOS1AP and YAP.

Reconfirming findings by our group, I performed a western blot showing NOS1AP isolated from HEK293T cells did in fact interact with YAP. Knowing NOS1AP is a highly spliced protein (Clattenburg et al., 2015) I then wanted to determine if YAP could associate with any of the isoforms of NOS1AP, namely NOS1APc, d, or e. I precipitated NOS1APc with the NOS1APc antibody or a pre-immune antibody. The NOS1APc antibody was able to precipitate YAP. This suggested that the extended isoforms of NOS1AP are able to associate with components of the Hippo pathway.

I then attempted to further characterize the interaction between NOS1AP and YAP by transfecting HEK293 cells with cDNA's encoding YFP-NOS1APa or YFP-NOS1APc in the presence of cDNA encoding flag-YAP. YFP-NOS1APa and Flag-Yap co-localized

at the cell membrane, where similar co-localization was detected for YFP-NOS1APc and flag-YAP. Based off our data, there is a better co-distribution between NOS1APc with YAP than with YAP and NOS1APa. NOS1APa contains a PTB domain and a PDZ-binding motif; we next wanted to determine if any of the known domains were responsible for the association with YAP. Our findings suggested the ΔPTB region of NOS1AP as being the region that is important for the association with YAP and that there may be other intermediary proteins involved in their association as NOS1AP and YAP do not overlap exclusively.

Previous studies revealed YAP plays a role in neurogenesis in the developing spinal cord; we then sought out to determine whether NOS1AP was localized in the spinal cord and whether NOS1AP played a functional role in the developing spinal cord. In the developing spinal cord, NOS1AP was restricted to the midline. NOS1APc was detected in the developing floor plate, roof plate and mid ventricular zone in E11.5 spinal cord.

NOS1APc associates and shows co-localization with YAP1 and is found in proliferating spinal cord cells, and has been shown to affect the proliferation of progenitor cells in the spinal cord. Next we turned to an *in ovo* system and overexpressed NOS1APc and monitored the expression of Sox2, a marker of proliferating cells. Embryo's electroporated with a cDNA encoding YFP-NOS1APc revealed unilateral expression of YFP and Sox2 staining revealed expression in the midzone, similar to the control sections. Small Sox2^{+ve} rosettes formed surrounding

some of the YFP-NOS1APc expressing cells in the lateral region of the spinal cord. These were not detected on the control half of the spinal cord.

4.2 NOS1AP isoforms in spinal cord and development

It is known that NOS1AP has various other isoforms. Clattenburg et al. identified in 2015 there were endogenous isoforms of NOS1AP, being NOS1APa, -c, -d, -e, and –f thus far with data suggesting potential for more. NOS1APa is commonly known as being the isoform referred to as NOS1AP or CAPON. Through proteomic screens, NOS1APc was the next isoform identified endogenously. The screens showed an isoform sharing the functional PTB domain with NOS1AP (NOS1APa from here on), while containing a 30kDa C-terminal extension that is absent from NOS1APa (Clattenburg et al., 2015).

Having observed that both NOS1APa and NOS1APc could interact with YAP, we further confirmed the co-distribution of isoforms, NOS1APa and NOS1APc individually with YAP through cell transfection and immunohistochemistry. My experiments indicated there might be other intermediary proteins involved in their association, as NOS1AP and YAP do not overlap exclusively.

Determining NOS1AP was endogenously located within the spinal cord through pan-NOS1AP antibody supported the idea that NOS1AP could be playing a regulatory role in the development of the spinal cord. NOS1AP is detected in the developing spinal cord along the midline in an E18.5 mouse embryo.

To begin to determine whether or not all of the NOS1AP isoforms were also present endogenously in the spinal cord, specifically the developing spinal cord, mouse spinal cords at different developmental stages were examined using a NOS1APc specific antibody. From this we were able to determine, consistent with mRNA studies, in the developing spinal cord of an E11.5 mouse NOS1APc resided in the floor plate, roof plate and mid proliferative zone. More specifically, NOS1APc had membrane localization and cell extensions that reached from the marginal zone to the pial surface. NOS1APc was also detected in cells that were dividing. Together, this suggests that NOS1AP proteins are expressed in the developing spinal cord. What was particularly interesting was the expression of NOS1APc in the progenitor regions of the spinal cord, and more specifically the dividing cells.

It is possible that the NOS1APc expression pattern in the progenitor domains of the developing spinal cord is one, or a combination of the two other isoforms in addition to NOS1APc, as it has been shown the NOS1APc antibody also identifies NOS1APd and NOS1APe (Clattenburg et al., 2015). The antibody created to identify NOS1APc recognizes the unique C-terminal and the PTB domain, all of which NOS1APd and NOS1APe contain, thus not as specific as originally intended. The two isoforms, NOS1APd and NOS1APe result from a splicing event that fuses amino acid 415 of Rattus NOS1APa with the unique C-terminal region in NOS1APc. NOS1APe has an extra 5-amino-acid insert in its PTB domain making it different from NOS1APd

(Clattenburg et al. 2015). Further study is required to identify the individual expression patterns of NOS1APc, NOS1APd and NOS1APe in the developing spinal cord. From there further information could be gathered to identify which isoform, if not all, of the three are involved in having a potential role in the regulation of development of the spinal cord through the Hippo pathway.

Overall, the expression pattern of NOS1APc in the developing spinal cord in addition to the interaction NOS1APc has with YAP, lead us to believe that perhaps NOS1APc plays a role in the development of the spinal cord acting through the Hippo pathway.

4.3. NOS1APc over expression in the developing Spinal Cord

We were able to show that NOS1APc is in the developing spinal cord, and you can see the expression pattern of NOS1APc resides within the progenitor domains, specifically in roof plate, floor plate and mid ventricular zone. In addition to being located in proliferative progenitor domains, NOS1APc was also detected in dividing cells. It is known that as the spinal cord develops it is organized into specific dorsoventral progenitor domains that later differentiate into specific neuron types, be it interneurons or motorneurons. Different signaling pathways are known to be involved in this differentiation process (Jessell, 2000).

When overexpressed in the developing avian neural tube, NOS1APc was widespread along the dorsoventral axis, residing in regions outside of the ventricular progenitor domain. More specifically, NOS1APc was in cells spanning the entire width of the

spinal cord, from the marginal zone to the pial surface. We saw in all of our animals overexpressing NOS1APc post electroporation, small Sox2^{+ve} rosettes formed surrounding some of the YFP-NOS1APc expressing cells in the lateral region of the spinal cord. The most pronounced rosette formation was in the animal surviving 2.5 days post electroporation. When magnified, some of the cells expressing Sox2 also expressed NOS1APc, though there were many cells expressing only Sox2 or NOS1APc. All of the Sox2^{+ve} rosette formations reside near or overlap the overexpression of NOS1APc.

It is known that as neural progenitor cells divide in the ventricular progenitor zone, they eventually receive signals to differentiate and migrate into their perspective post-mitotic neuron domain within the spinal cord. As the overexpression of NOS1APc was fluid throughout the dorsal and ventral areas of the spinal cord it may be that NOS1APc is not involved in the signaling pathways specifically responsible for the dorsoventral organization of the developing spinal cord. As the rosette formations were consistent in both the ventral and dorsal regions of the spinal cord, it is possible NOS1APc could be affecting the signaling cascades that determine when a neural progenitor cell will exit cell cycle and differentiate.

YAP, acting through the Hippo pathway, could be playing a role in regulating the timing of progenitor cell cycle exit. Cao et al. showed in 2008 the overexpression of YAP1 increased activation of TEAD, the downstream transcription factor, increased neural progenitor proliferation, and inhibited differentiation. They showed that this

increase in activation of the Hippo pathway inhibited the expression of neurogenic bHLH factor *NeuroM* (Cao et al., 2008) and also affected Cyclin D1 signaling. Cyclin D1 is known to play a role in length of the G1 cell cycle phase, which is known to determine whether or not a cell will continue to proliferate or exit cell cycle and differentiate (Hardwick et al., 2014). When YAP1 and TEAD were suppressed, *NeuroM* expression increased, which lead to premature neuronal differentiation. It is also speculated the overexpression of YAP/TEAD results in disruptions in adherens junctions and a loss of neuroepithelial cell polarity. This results in the overexpression of $Sox2^{+}$ cells, with rosettes also forming in ectopic regions of the developing spinal cord (Cao et al. 2008). It is possible that NOS1APc could be acting through the Hippo pathway to intrinsically regulate cell cycle exit in proliferating neural progenitor cells, while playing a role in maintaining cell polarity.

NOS1APc may also play a regulatory role through other signaling pathways altogether known to play a role in cell cycle exit and dorsoventral patterning. NOS1APc is detected within the roofplate and floorplate of the developing spinal cord, in addition to its expression in the mid ventricular zone, known to be the main organizing regions of the developing neural tube. It is known that Notch and Wnt signaling pathways play a role in maintaining a progenitor fate in neural progenitor cells in the beginning stages of spinal cord development. Increased activation of either of these signaling pathways has lead to an expansion of the neural progenitor population (Lardelli el al. 1996; Chenn and Walsh 2002; Megason and McMahon 2002). It may be that NOS1APc is acting through other pathways known to reside

within the specialized FP and RP regions of the developing spinal cord to regulate neural progenitor cell differentiation.

NOS1APc was also observed to reside in the same location, though not within the same cells expressing Sox2 exclusively. NOS1APc could be having extrinsic effects on neighboring cells within the ventricular zone of the developing spinal cord. It was observed that NOS1APc is expressed in cells with processes that extend the entire width of the neural tube. It is well studied in the cortex that Radial Glial cells (RGCs) play a functional role in cell migration of newly differentiated neuronal cells. RGCs provide a conduit of transportation for some cells in the developing cortex, allowing them to attach and use the extended processes of the RGC to relocate to the specific region where they'll reside during the rest of development (Paridaen and Huttner, 2014). Though not as well studied, it is speculated that specific differentiation cells use RGCs to migrate during spinal cord development as well. It is possible that NOS1APc is expressed within RGCs in the neural tube, and plays a regulatory role within migration. Further research is required to determine which developmental process NOS1APc is regulating, be it progenitor cell proliferation, cell cycle exit or neuronal migration within the spinal cord.

4.4. NOS1AP, The Hippo Pathway, and YAP

The development of the spinal cord is particularly important, as it is the caveat for sensory-motor circuits to be correctly organized and transmitted. The Hippo pathway is known to play a role in tissue development in various different parts of

the body, and more recently has been shown to play a role in the development of the spinal cord. Cao et al, discovered in 2008 when YAP1 is overexpressed through electroporation in the developing chick spinal cord between HH12-14 it causes an increase in cell proliferation, denoted by an increase in Sox2 expression. In addition to an increase in Sox2 expression, these expressing cells were also identified due to the specific rosette formations the $Sox2^{+ve}$ cells formed in the ectopic regions of the spinal cord. It was also reported the increased expression of YAP1 causes an increase in the activation of the target transcription factor, TEAD. This study was the first insight into the role the Hippo pathway might play in the formation of progenitor domains, and neuron differentiation in the developing spinal cord (Cao et al., 2008).

In 2011, Hateren et al. further showed the role the Hippo pathway plays in regulating progenitor cell proliferation and differentiation in the developing spinal cord. By knocking out FatJ using a FatJ RNAi construct through in *ovo* electroporation, they were able to show FatJ acts via the Hippo pathway mediator YAP1 to regulate the size of the dp4-vp1 progenitor cell pools. When FatJ was knocked out, the progenitor cell pool increased, thus increasing overall width of the spinal cord (Van Hateren et al., 2011). The knockdown of FatJ ultimately had an effect on the size of the differentiated $Lim1^{+}/Lim2^{+}$ interneuron pool. Though our data is only preliminary, it suggests when overexpressed in the developing chick neural tube NOS1AP creates a similar phenotype reported by the overexpression of YAP1, which is similar to the phenotype observed when FatJ is knocked out.

NOS1AP overexpression resulted in Sox2 rosette formations, though to a lesser extent than the rosette formations caused by YAP1 overexpression in the spinal cord of the developing chick spinal cord.

The increase $Sox2^{+}$ expression is indicative of an expanded progenitor pool, as detected by the rosette formation in the ectopic region of the developing spinal cord of electroporated embryos. Whether or not NOS1AP acts through the Hippo pathway mediator YAP1 has yet to be determined. Contrary to the phenotype produced by the inhibition of FatJ, our data suggests NOS1APc has the same affect on cell proliferation irrespective of progenitor domain.

4.5.What is the role of NOS1AP?

There is some speculation on what potential role NOS1AP plays within the developing spinal cord and the Hippo pathway. Clattenburg et al., (2015) recently showed that the overexpression of NOS1AP in MCF7 and HEK293T cells increased the phosphorylation of the Hippo pathway downstream target YAP1. In addition to increasing the level of phosphorylated YAP1 sequestered into the cytoplasm, it was also observed that the overexpression of NOS1AP resulted in decreased levels of TEAD activation. This would suggest that the overexpression of NOS1AP decreases proliferation by inhibiting the ability of YAP1 to travel into the nucleus and activate the target transcription factor, TEAD (Clattenburg et al., 2015).

Interestingly, when testing which region of NOS1AP could be responsible for the

observed interaction with YAP, our imaging indicated ΔPTB had a more specific co-distribution with YAP than did the PTB domain. We observed that the PDZ binding motif domain of NOS1AP may be the responsible domain for the interaction with YAP. These findings are inconsistent with data recently published by Clattenburg et al., (2015), who showed the PTB domain of NOS1AP is suspected to regulate YAP1 and be responsible for the increase in phosphorylation of YAP through Lats1. Reasons for the inconsistencies between my results and the results published by Clattenburg et al. in 2015 could primarily be due to the difference in constructs being used.

Clattenburg et al., (2015) showed through a luciferase assay when myc-PTB is expressed with or without Flag-YAP in the presence of the TEAD reporter, the reporter (TEAD) activity decreased. This indicates that the PTB domain is responsible for the affect NOS1AP has on the Hippo pathway, specifically the increased phosphorylation of YAP. We were unable to detect an interaction between YFP-NOS1AP and Flag-YAP when exogenously expressed in HEK293T cells at the same time though we saw co-distribution of both NOS1APa and YAP and NOS1APc and YAP. The experiments performed by Clattenburg et al., (2015), and our lab co-expressed NOS1AP and YAP at the same time, though the NOS1AP constructs used were different. It may be that the YFP-tag within the cDNA vector is driving the NOS1AP construct to specific regions in the cell, rendering it not possible for YAP and NOS1AP to interact. It has been speculated that fluorescent protein tags have the ability to produce localization artifacts not true to the proteins actual

endogenous cellular location (Margolin, 2012). It is possible when we express YFP-PTB or YFP-ΔPTB in the presence of Flag-YAP in HEK293T cells the YFP-tag was affecting the intracellular localization of the PTB domain, thus affecting ability of the PTB domain to co-distribute with YAP.

It was observed in the developing avian spinal cord the results of over expressing NOS1APc are also fairly contradicting in comparison to what was reported in the study conducted by Clattenburg et al., (2015). Based on preliminary observation, overexpression of NOS1APc appeared to cause an increase in neural progenitor cells that not only were still proliferating, but they also formed rosettes in areas of the spinal cord where differentiated cells typically reside. Due to the low number of animals obtained in this data set, it could be the phenotype in the animals successfully electroporated isn't portrayed in being a true phenotype.

It was observed that the number of animals that did not survive post electroporation was significantly higher in comparison to those that did survive and positively expressed YFP-NOS1APc. Cao et al. showed in 2008 when YAP1 and TEAD were inhibited in the neural tube, apoptosis was increased. In YAP mutant mice, embryos did not live past E8.5 and were riddled with widespread defects (Morin-Kensicki et al., 2006). There is a possibility that NOS1AP functions to inhibit YAP, potentially leading to increased apoptosis of neural progenitor cells, and ultimately death, which may explain the large number of animals that did not survive during this study.

An alternate possibility could be a result of the *in ovo* electroporation technique itself. When optimizing the technique there are a number of parameters that need to be taken into consideration to ensure none of the results seen in the animal are a product of the technique (Croteau and Kania, 2011). Working with a technique involving voltage and current passing through an animal, it is necessary to optimize the correct voltage/current the animal is receiving. If the electrodes are too close to the animal, or the voltage is creating too high of a current the animal is receiving, the animal will likely die, or experience morphological defects (Croteau and Kania, 2011). It was also observed animals between HH14-16 have a fairly developed circulatory system. If the electrodes are placed near, or on any of the developing veins, the vein is severed during the voltage pulses, and the animal bleeds out, typically resulting in death or having severe developmental defects. Finally, if during DNA injection the glass needle pierces the animal below the neural tube, there is potential for growth defects to occur as a result of the laceration.

In addition to mechanical errors than can occur as a result from the technique, it has been reported when DNA is diluted in TE buffer versus water, it has the potential to cause defects in cell polarity and cell adhesion in the neural tube, showing the potential to produce phenotypes within the spinal cord (Croteau and Kania, 2011). Further optimization of the *in ovo* electroporation technique for this construct is necessary to answer whether or not the phenotype we observed was a result of the technique.

4.6 Future Directions

We were able to identify NOS1AP is not only located in the spinal cord, but as well it interacts with YAP. This study gave a brief insight to a potential role NOS1AP could play acting through the Hippo pathway in spinal cord development. I believe the next step to fully elucidate what role NOS1AP might play, as contradictory results have been displayed, would be to optimize the electroporation technique further, and develop functional constructs for all of the known NOS1AP isoforms residing endogenously in the spinal cord.

In addition, an alternative way to see whether or not NOS1AP or any of the isoforms play a functional role in the development of the spinal cord using *in ovo* electroporation could be accomplished by creating a morpholino for each of the isoforms know to be in the spinal cord to date.

Dependent on the whether or not there is a phenotype displayed as a results of electroporation experiments, identifying whether NOS1AP acts through the Hippo pathway by tracking YAP and TEAD activity, be it activation or phosphorylation would give more insight on the role NOS1AP may play in spinal cord development. The amount of animals that died greatly outweighed those that survived and expressed YFP-NOS1APc. Potential reconstruction of the plasmid containing a more specific chick promoter region, such as pMiwIII, pRc/CMV, or pCAGGS could potentially increase the number of animals positively expressing the YFP-NOS1APc

construct, as well as other isoform constructs (Odani et al. 2009).

4.7 Summary

We have identified that both NOS1AP and NOS1APc are located in the developing spinal cord as well as the adult spinal cord. We were able to show that at different time points NOS1AP is located in different regions of the developing spinal cord. This was also observed for NOS1APc. It was observed that NOS1APc was located in dividing cells within the developing spinal cord.

The regulatory role of NOS1AP and its isoforms is still largely unknown within the developing spinal cord. Previous studies revealed YAP plays a role in neurogenesis in the developing spinal cord, this study showed NOS1AP and YAP interact in HEK293T cells, MCF7 cells and within the rodent brain.

Our study showed NOS1AP potentially plays a regulatory role in progenitor cells proliferation and cell cycle exit. Overexpression of NOS1APc in the developing spinal cord of avian embryos HH14-16 caused an overexpression of $SOX2^{+}$ expressing cells that produced rosette formations in the ectopic region of the developing spinal cord. This phenotype was inconsistent with results detected in the lab when NOS1AP is overexpressed in HEK293T and MCF7 cells, as it was reported to cause YAP phosphorylation and decrease proliferation. Further investigation into role of NOS1AP and NOS1AP isoforms in the spinal cord is required.

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