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Understanding the Mechanisms of Floor Plate Specification in the Vertebrate Midbrain and its Functions during Development

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Understanding the Mechanisms of Floor Plate Specification in the Vertebrate Midbrain and its Functions during Development

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Dedication

I dedicate this dissertation to my parents for their continual love and support throughout the years.
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I would like to especially thank my wife. I would definitely not be the person I am today without her continual love, support and understanding.
We have previously shown that the arcuate organization of cell fates within the ventral midbrain critically depends upon the morphogen, Sonic Hedgehog (SHH), which is secreted from a signaling center located along the ventral midline, called the floor plate (FP). Thus, it is ultimately the specification of the FP that is responsible for the patterning and specification of ventral midbrain cell fates. Interestingly, we have found that the chick midbrain FP can be divided into medial (MFP) and lateral (LFP) regions on the basis of gene expression, mode of induction and function. Overexpression of SHH alone is sufficient to recapitulate the entire pattern of ventral cell fates, although remarkably it cannot induce MFP, consistent with the observation that the MFP is refractory to any perturbations of HH signaling. In contrast, overexpression of the winged-helix transcription factor FOXA2/HNF3β robustly induced the MFP fate
throughout ventral midbrain while blocking its activity resulted in the absence of the MFP. Thus, by analyzing the differences between SHH and FOXA2 blockade and overexpression, we were able to attribute functions to each the LFP and the MFP. Notably, we observed that FOXA2 overexpression caused a bending of the midbrain neurepithelium that resembled the endogenous median hinge-point observed during neurulation. Additionally, FOXA2 misexpression led to a robust induction of DA progenitors and neurons that was never observed after SHH expression alone. In contrast, we found that all other ventral cell types required HH signaling directly, at a distance and early on in the development of the midbrain when its tissue size is relatively small. Additionally, HH blockade resulted in increased cell-scatter of the arcuate territories and in the disruption of the regional boundaries between the ventral midbrain and adjacent tissue. Thus, we bring new insight into the mechanism by which midbrain FP is specified and ascribe functional roles to its subregions. We propose that while the MFP regulates the production of dopaminergic progenitors and the changes in cell-shape required for bending and shaping the neural tube, the LFP appears to be largely responsible for cell survival and the formation of a spatially coherent pattern of midbrain cell fates.
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FOXA2 is indirectly required for the specification of the red nucleus and the oculomotor complex

Gene expression suggests that dopaminergic neurons originate from within the MFP

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Chapter 1: Introduction

During development, specialized groups of cells, termed signaling centers, secrete instructive signals that are required for the appropriate specification and organization of adjacent tissues. The floor plate, located along the ventral midline of the neural tube, is a signaling center that is critically important in the development of the central nervous system whose anatomy and function have been studied for over a century (His, 1888; Kingsbury, 1920). The floor plate is a principal source of the morphogen sonic hedgehog (SHH) that can specify multiple neural cell types in a dose-dependent manner (Marti et al., 1995a; Ericson et al., 1997; Patten and Placzek, 2000). Additionally, the floor plate is a source of multiple guidance cues (e.g. Netrins) required for the correct coordination of axonal trajectories (Colamarino and Tessier-Lavigne, 1995; Charron et al., 2003). Thus, it is ultimately the specification of the floor plate that is responsible for the overall pattern of cell fates within the midbrain. Here, we ask the overarching questions, 1) what is the requirement of HH signaling in the specification of ventral midbrain cell fates? and 2) what are the functions of midbrain floor plate?

The arcuate organization of ventral cell fates within the midbrain is dependent upon Sonic Hedgehog

The ventral midbrain, or tegmentum, is home to several clinically important nuclei. They include the red nucleus, the oculomotor complex (OMC) and the dopaminergic neurons of the ventral tegmental area (VTA) and substantia niagra (SN). While some effort has been spent on the development of the red nucleus and the OMC,
the overwhelming majority of work in the ventral midbrain is focused on understanding midbrain dopaminergic neuron (DA) induction and function. DA neurons of the VTA are involved in the behavior-reward pathway, whose secretion of dopamine paves the way for most addictive behaviors and those essential for life such as satiety and sex drive (Chao and Nestler, 2004). In contrast, DA neurons of the SN also modulate fine motor control, whose degeneration is the principal cause of Parkinson’s Disease. Thus, a better understanding of the developmental mechanisms involved in the specification of these neuronal cell types may lay the foundation for future therapeutic strategies.

Early on in the development of the midbrain, ventral cell fates are organized into longitudinal stripes that are arrayed in parallel to the ventral midline (Fig. 1.1; Agarwala et al., 2001; Sanders et al., 2002). These stripes of cell fates appear in a medial to lateral sequence, taking on an arcuate trajectory whose curvature mirrors the shape of SHH expression within the floor plate. Misexpression of SHH within the ventral midbrain recapitulates the entire pattern of ventral cell fates, showing for the first time in vivo that a single source of SHH was sufficient to induce multiple cell fates (Agarwala et al., 2001). Interestingly, midbrain arcs manifest themselves as physical ridges along the ventricular surface of the midbrain neurepithelium, identifiable through scanning electron microscopy (Ragsdale and Lumsden, 1995). It was thus theorized that the nuclear organization of midbrain cell fates was organized early within the context of the arcuate pattern of midbrain progenitors (Agarwala and Ragsdale, 2002; Sanders et al., 2002).
Figure 1.1. The arcuate pattern of cells fates within the ventral midbrain

Left. A sagittal view of an embryonic day (E) 5 chicken embryo, revealing the relative orientation of the midbrain (blue) with respect to the hindbrain, (green), forebrain (orange) and the eye (purple). Right. When dorsal midbrain is removed and viewed in wholemount with the ventricular surface facing the viewer, stripes of cell fates with arcuate trajectories (midbrain arcs, blue, purple, yellow) are observed that run parallel to the ventral midline source of SHH (brown, light brown).
Mutant mice lacking a functional Shh or Smo (an obligate effector for all HH ligands) gene lose all ventral cell fates and tissue in the midbrain by E9, suggesting its requirement in the proper specification of midbrain cell types (Fogel et al., 2008). However, Shh is present much earlier in the developing embryo, including the node and its derivatives that affect midbrain patterning (Lawson et al., 2001; Patten et al., 2003; Aglyamova and Agarwala, 2007). Therefore, the absence of ventral cell fates in Shh−/− mice may be due to an indirect effect of altering early developmental signaling centers. Precise blockade of HH activity in the midbrain as ventral cell types emerge would lead to a better understanding of the temporal and spatial requirement of HH signaling on ventral cell fate specification in addition to any functions HH may have later in development that null mutant analyses do not allow.

The Hedgehog signaling pathway

Hedgehog (HH) is a secreted ligand that binds to its receptor and negative regulator, PTC1 (Marigo and Tabin, 1996; Stone et al., 1996; Ingham and McMahon, 2001; Hooper and Scott, 2005). Once the HH ligand is bound to PTC1, its constitutive blockade of the transmembrane protein Smoothened (SMO) is lifted (Alcedo et al., 1996; Akiyama et al., 1997). A complex cascade downstream of SMO then converges to activate the GLI family of transcription factors (GLI1, GLI2, GLI3), by repressing their cleavage into repressor forms (GLI2, GLI3) (Aza-Blanc et al., 1997; Dai et al., 1999; Sasaki et al., 1999; Litingtung and Chiang, 2000; Bai et al., 2004). In the absence of an HH ligand, PTC1 maintains its constitutive blockade on SMO, allowing the proteolytic processing of GLI2 and GLI3 into their repressor forms. Interestingly, the inhibition of SMO by PTC1 is indirect and non-stoichiometric (Ingham et al., 2000).
The role of Hedgehog signaling in neural cell-fate specification

The ability of SHH to pattern tissue and specify multiple cell fates is best characterized in the spinal cord (Jessell, 2000). Several gain- and loss-of-function experiments have demonstrated that SHH is both necessary and sufficient for the specification of ventral cell fates (Chiang et al., 1996; Briscoe and Ericson, 2001; Zhang et al., 2001a). Additionally, SHH is sufficient to induce the ventral cell types of the spinal cord in a dose-dependent manner (Jessell, 2000). These findings led to a very attractive model whereby cells acquire their identity by crossing varying thresholds of SHH exposure. Cells in close proximity to the source of SHH (FP) received high levels of SHH protein while cells located more dorsal receive increasingly lower amounts of SHH (Figure 1.2; Jessell, 2000). The secretion of SHH from the floor plate to act directly and at a distance to specify ventral cell fates was later validated by misexpression of a mutant form of Ptc1, the receptor for the SHH ligand (Marigo et al., 1996c; Briscoe et al., 2001). The mutant receptor, Ptc1\(^{\Delta}\)loop\(^2\), lacks its second extracellular domain that is required to bind to the HH ligand. Thus, the mutant constitutively inhibits Smo function in a cell-autonomous manner regardless of the presence of any HH ligand (Briscoe et al., 2001). The direct action of SHH at a distance to specify spinal cord cell fates was also observed in mice that were mosaic for the Smoothened gene, an obligate effector of the HH signaling pathway. Cells lacking Smoothened between 15-20 cell diameters away from the source of SHH become dorsalized, suggesting that these cells require a HH ligand at this distance for their proper specification. Similar distances are also observed in the developing mouse limb bud (~20) and fly wing (12-15) (Wijgerde et al., 2002; Harfe et al., 2004; Tabata and Takei, 2004).
Figure 1.2. SHH is sufficient to specify multiple cell fates in a concentration-dependent manner

**Left.** SHH, secreted from the floor plate and underlying notochord, acts to specify multiple spinal cord cell fates. The concentration of SHH protein is highest near the source of SHH (FP) and is least concentrated far away. **Right.** Crossing various thresholds of total exposure to the SHH ligand, incorporating both the amount of ligand and the duration, is thought to provide positional information for the creation of multiple cell fates. *Abbreviations:* D: Dorsal; FP: floor plate; NC: notochord; V: ventral. Image based on figures from (Briscoe, 2009).
Hedgehog signaling regulates cell affinities and the cohesion of tissues

Although all ventral cell fates are lost in mice lacking Shh, the majority are rescued in Shh\textsuperscript{-/-};Gli3\textsuperscript{-/-} double mutants, but are not organized into discrete domains (Chiang et al., 1996; Litingtung and Chiang, 2000; Bai et al., 2004). These observations suggest additional molecules may be involved in ventral cell fate specification and that SHH is required for the cohesion of neural progenitors. Interestingly, in the chick talpid\textsuperscript{2}, a naturally occurring HH pathway mutant, all ventral cell types are specified, but the normal arcuate organization of cell fates is disrupted, appearing in a scattered fashion (Agarwala et al., 2005). Additional observations supporting a role for Hedgehog in regulating cohesion of tissues come from the fly wing and abdominal ectoderm (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence et al., 1999). In each tissue, differential responsiveness to the HH signal instructs cells to segregate and adhere as a group, creating compartments. The separation of adjacent compartments creates a sharp, lineage restriction boundary that can act as a signaling center (Garcia-Bellido et al., 1973; Morata and Lawrence, 1975; Blair, 1992; Lawrence et al., 1999). These results implicate HH signaling in the establishment of tissue boundaries and in the maintenance of a spatially coherent pattern (Dahmann and Basler, 1999). Recently, HH signaling has also been implicated in the maintenance of orthogonal signaling centers in the vertebrate limb and the MHB of the neural tube (Aoto et al., 2002; Khokha et al., 2003; Blaess et al., 2006). However, whether regulation of boundaries is a general feature of HH action among vertebrates is not yet known.
The role of Hedgehog signaling in the development of the floor plate

Within the last 20 years, a significant effort has been made to understand the development, architecture and patterning activities of floor plate in amniotes and anamniotes leading to substantial controversy in the field concerning the origin of the FP (Placzek and Briscoe, 2005). Much of the controversy stems from the fact that the origin and mode of floor plate induction differs within and among studied organisms. Mutant mice lacking functional \( shh \), its signaling components (\( smo \) and \( gli2 \)) as well as one of its transcriptional targets (\( foxa2 \)) fail to form a floor plate while mice mutant for \( patched1 \), a negative regulator of Hedgehog (HH) signaling, possess a significantly enlarged floor plate (Ang and Rossant, 1994; Weinstein et al., 1994; Chiang et al., 1996; Goodrich et al., 1997; Wijgerde et al., 2002; Bai et al., 2004). These findings were complemented by gain of function experiments in mouse and chick in which notochord transplantations or explanted naïve neural tissue exposed to recombinant shh induced floor plate markers (van Straaten et al., 1985; Echelard et al., 1993; Roelink et al., 1994; Marti et al., 1995a). Together, these experiments lead to the classical model of floor plate induction whereby Shh, secreted from the notochord, activates downstream effectors in the overlying neurectoderm, which in turn activates \( shh \) itself, responsible for generating the diversity of cell types located within the ventral neural tube (Fig. 1.3; Jessell and Dodd, 1990; Dodd et al., 1998; Placzek et al., 2000). However, recent work has challenged this idea implicating additional signaling molecules in floor plate specification in part due to the observation that floor plate can be divided into subcomponents on the basis of gene expression, mode of induction and embryonic origin (Odenthal et al., 2000).
Floor plate can be divided into medial and lateral regions

Initial evidence supporting the division of floor plate into medial (MFP) and lateral (LFP) subregions has come from studies in zebrafish, where gene expression patterns and mechanisms of induction differ from those in the mouse and chick (Appel, 2000; Strahle et al., 2004). Interestingly, unlike in \textit{shh} \textsuperscript{-/-} mice, MFP, but not LFP can develop in \textit{syu} and \textit{smu} zebrafish mutants lacking HH signaling (Schauerte et al., 1998; Chen et al., 2001; Karlstrom et al., 2003). Conversely, \textit{cyc}, \textit{oep} and \textit{sur} mutants, which constitute components of the Nodal signaling pathway fail to form MFP even though \textit{shh} is present in the notochord (Strahle et al., 1996; Odenthal et al., 2000). However, the loss of floor plate in \textit{cyc} mutants can be rescued by the winged/helix transcription factor, Foxa2, a target of the Nodal signaling pathway that activates \textit{shh} in the floor plate (Strahle et al., 1993; Rastegar et al., 2002). Moreover, nodal signaling through the activity of Smad2 and FoxH1 directly activates \textit{shh} expression in the floor plate (Muller et al., 2000). These data support a two-step model for floor plate induction whereby Nodal signaling from the notochord activates \textit{shh} through the action of Foxa2 in MFP and Shh in turn induces LFP (Fig. 1.3).

Sonic Hedgehog may cooperate with other signals to specify floor plate

The requirement of signals in addition to Shh in the specification of MFP is corroborated by observations in chick through tissue grafting and cell lineage analysis. Interestingly, separate origins exist for anterior and posterior floor plate (Schoenwolf et al., 1989b; Schoenwolf and Sheard, 1990). During gastrulation when prechordal
Figure 1.3. Mechanisms of floor plate specification in amniotes and anamniotes

Left. Floor plate induction in amniotes (in the mouse and chick) is principally thought to occur through a feed-forward mechanism where SHH is secreted from the notochord to induce its own expression in the overlying neurectoderm through the activation of transcription factors such as FOXA2. Interestingly, recent data from the chick spinal cord suggests that floor plate specification may occur more like what is observed in zebrafish. Right. In zebrafish, Nodal signaling is required from the notochord to activate both Shh and Foxa2 in the medial floor plate. Shh secretion from the MFP is then necessary for the specification of the lateral floor plate. These figures were adapted from Strahle et al., 2004.
mesoderm is migrating rostrally, the overlying ectoderm anterior to Henson’s node, called ‘area a’, is briefly exposed to the synergistic action of NODAL and SHH signaling that then take on a floor plate fate. These newly induced floor plate cells migrate both in an anterior and posterior direction to occupy the ventral midline of the diencephalon, midbrain and rostral hindbrain (Schoenwolf and Sheard, 1990; Patten et al., 2003). This is in contrast to more posterior regions of the neural tube whereby cells derived from within the organizer are left behind as the node regresses caudally and integrate into either the floor plate or the notochord (Schoenwolf et al., 1989a; Catala et al., 1996).

Evidence in the chick for the subdivision of floor plate into medial and lateral regions

Floor plate cells derived from either the node or ‘area a’ eventually occupy a medial portion of floor plate and the genes that they express and their cell biology differ from floor plate cells located more laterally (His, 1888; Kingsbury, 1920; Schoenwolf et al., 1989a; Schoenwolf and Sheard, 1990; Charrier et al., 2002). Posterior floor plate in chick can be divided into an MFP region expressing SHH, FOXA2 and lacking expression of SOX1, a pan neural marker, and an LFP region that expresses NKX2.2, SHH and SOX1 (Charrier et al., 2002). Additionally, cells located in the MFP exhibit a polarized, wedge shaped cell morphology and contain basally located nuclei, whereas cells located in the LFP maintain a characteristic pseudostratified columnar neurepithelial organization (His, 1888; Kingsbury, 1920; Catala et al., 1996). Intriguingly, the full floor plate gene expression pattern can be ectopically induced after grafting either a quail notochord or floor plate to a more dorsal region of a chick neural tube.
including the morphological tissue shape change mirroring that of the endogenous ventral hinge point whereas implantation of SHH-producing cells alone is only sufficient to induce LFP (Charrier et al., 2002). Although controversial, it is believed that posterior floor plate and notochord in chick is node-derived and that these cells express an as yet unidentified molecule necessary to act in cooperation with SHH to elicit the full floor plate pattern (Catala et al., 1996; Placzek et al., 2000; Charrier et al., 2002). It is possible that the MFP may require Nodal signaling as observed in zebrafish or the modulation of BMP signaling as in the rostral diencephalic ventral midline (Dale et al., 1997; Odenthal et al., 2000; Charrier et al., 2002).

**FOXA2 function in floor plate development**

Much of the differences in inductive mechanisms of floor plate between species centers on the regulation of FOXA2. In zebrafish and mouse, Foxa2 can directly bind to and activate transcription of *ntn1* and *shh*, under the control of Nodal in zebrafish and Shh signaling in mouse (Epstein et al., 1999; Muller et al., 1999; Rastegar et al., 2002; Jeong and Epstein, 2003). However, Foxa2 can act both upstream and downstream of Shh, making it difficult to identify independent functions for each (Ruiz i Altaba et al., 1995; Chang et al., 1997). Indeed, misexpression of *SHH* and *foxa2* in midbrain cause the transcriptional activation of each other and produce overlapping, but not identical phenotypes (Sasaki and Hogan, 1994; Ruiz i Altaba, 1998; Agarwala et al., 2001). Taken together, it remains unclear how these two signaling molecules cooperatively function to specify floor plate, in particular how they partition floor plate into medial and
lateral regions and how they regulate the territorial expansion observed through midbrain development.

**Midbrain floor plate is neurogenic**

Most of our understanding of the development of floor plate comes from studies focused on posterior spinal cord whereas those of more anterior regions have been limited (Placzek and Briscoe, 2005). As the origin and mode of induction differs between anterior and posterior regions of floor plate, so does their anatomy and function. Midbrain floor plate, in particular, is the source of oculomotor and dopaminergic neurons (Kittappa et al., 2007; Ono et al., 2007). Although both cell types are derivative of the floor plate, it is unclear whether their progenitors exist in a mixed population or differentiate from a common progenitor pool. Several intrinsic and extrinsic developmental control genes have been identified in the specification of dopaminergic neurons (\textit{LMX1A, LMX1B, NURR1, PITX3, SHH, FOXA2}), but relatively little is known about how oculomotor neurons of the midbrain are specified (Zetterstrom et al., 1997; Smidt et al., 2000; Maxwell et al., 2005; Andersson et al., 2006).

**The development of the median hinge-point during neurulation**

Neurulation is the developmental process by which a sheet of undifferentiated neural cells folds to create a neural tube. Defective neurulation leads to a failure of the neural tube to close, the second leading cause of all congenital deformities (Copp et al., 2003). Interestingly, while over 100 genes have been implicated in the closure of the
neural tube along the dorsal midline, almost nothing is known about the genetics of the formation of the hinge-point along the ventral midline (Copp et al., 2003). The first step during neurulation involves the folding of the neurepithelium at the midline of the embryo. This morphological process occurs in part due to changes in cell-shape of specific cells that are positioned along the midline. Originally cuboidal in shape, these cells become constricted at their apical surface and their nuclei translocate basally, ultimately causing a change to a wedge-shaped morphology. As a group, these wedge-shaped cells effectively create a hinge-point forcing the lateral regions of the neurepithelial sheet closer together (Schoenwolf and Smith, 2000).

In the chick, wedge-shaped cells are present within the MFP of the spinal cord, but not the LFP (Charrier et al., 2002). Interestingly, transplantation of the notochord or the MFP was sufficient to induce the wedge-shaped morphology in naïve lateral regions of the neural tube, whereas transplantations of the LFP or SHH-producing cells could not (van Straaten et al., 1985; Charrier et al., 2002). These observations suggest that the cell shape and tissue morphology within the MFP is controlled by signals other than SHH, that come from the notochord or the MFP itself. However, whether similar mechanisms operate during the development of the midbrain is unknown, as it has been suggested that the notochord is largely not involved in the induction of midbrain floor plate (Patten et al., 2003).
Chapter 2: Materials and Methods

Chick embryos

Fertilized Leghorn eggs (Gallus gallus) were purchased locally (Ideal Poultry, Texas) and incubated at 38°C in a forced draft-humidified chamber (G.Q.F. Mfg. Co.). The first day of incubation was designated as embryonic day 0 (E0). Embryos were staged according to criteria described by Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Egg windowing

In order to facilitate electroporation in ovo, working space needs to be created between the embryo and the shell of the egg. Best results are observed when eggs are incubated on their side, so that their longest axis is parallel to the floor. Chicken embryos develop on the surface of the yolk and rise towards the “top,” independent of egg orientation. Incubating the eggs on their side also creates working distance between the embryo and the lens of a dissecting scope through which it is viewed. The embryo normally develops close to the egg shell, so if one removes a portion of the egg shell, they are likely to destroy the embryo as well. To avoid this, albumin needs to be removed to effectively lower the embryo within the egg. First, two small holes are drilled into the egg shell with a scalpel; one at the top of the egg and another at its smaller side. Approximately 1-2.5 mL of albumin is removed (dependent upon the size of the egg) via needle and syringe from the egg prior to windowing and electroporation. A piece of transparent polyethylene tape (3M) is placed on top of the egg, covering both of the
holes. This allows for scissors to cut an opening on the top of the egg without cracking the rest of the shell. The exposed embryo can then be electroporated, re-covered with tape and placed back into the incubator until the desired stage is reached. Viability is typically best when the albumin is removed one day before electroporation.

In ovo electroporation

Electroporation was carried out with slight modifications from previously reported protocols (Agarwala et al., 2001; Agarwala and Ragsdale, 2002). Approximately 50-150 nl of plasmid DNA solution (1-3 μg/μl DNA, 0.02% Fast Green Dye (Fluka), 10mM Tris-HCl (Fisher Scientific) and 1mM EDTA (Fluka)) was injected (Pneumatic Pico Pump; WPI) through a glass microcapillary pipette (Sigma), tapered by a flaming micropipette puller (Sutter Instrument Co.), into the presumptive midbrain lumen of H&H stage 6-20 chicken embryos. Quickly after injection of the DNA solution into the midbrain lumen, a negative electrode (0.001” platinum wire, A-M Systems, Inc.) was inserted into the lumen of the presumptive midbrain while the positive electrode (0.002” platinum wire, A-M Systems, Inc.) was placed approximately 1 mm lateral to the right side of the midbrain. Three 25 ms, 5V square wave pulses were then delivered (Electro square porator ECM830; BTX). The directed current from the negative electrode inside the lumen to the positive electrode outside the lumen is thought to drive the negatively charged plasmid DNA into tissue that lay in between each electrode. After electroporation, embryos were recovered with tape and returned to the incubator for 1-7 days prior to collection for further analyses. Only 20% of the embryos electroporated between H&H 6-8 survived to E5. After harvesting, the embryos were submerged in 4% paraformaldehyde in
phosphate-buffered saline (PBS; Sigma) solution (PFA). Developmentally defective embryos were not electroporated and those that displayed any sign of injury from the electroporation were excluded from the study.

**Expression vectors used for electroporation in ovo**

**EGFP**

Embryos electroporated with Enhanced Green Fluorescent Protein (EGFP; EFX-EGFP) alone served as controls for those that were bilaterally-electroporated with our gene of interest. Additionally, EGFP was also coelectroporated with constructs enabling us to screen embryos through fluorescence detection. The EFX-EGFP construct was created by ligating the BamH1-Not1 fragment (800 bp) of pEGFPN1 (Clontech) into the plasmid EFX3C (Invitrogen). EFX3C utilizes the elongation factor-1α promoter and has previously been shown to robustly drive gene expression in the chick (Agarwala et al., 2001).

**Hedgehog signaling**

We manipulated Hedgehog signaling through the electroporation of Gli1 (EFX-hGli1), Ptc1Δloop2 (pCIG-Ptc1Δloop2), and SHH (XEX-SHH) containing expression vectors. EFX-hGli1 was constructed by ligating the EcoRV-Not1 fragment from human Gli1 (pBSSK-hGli1) into EFX3C (Kinzler et al., 1988). The construction of Ptc1Δloop2 and XEX-SHH have been described previously (Agarwala et al., 2001; Briscoe et al., 2001; Agarwala and Ragsdale, 2002). Briefly, the second large extracellular loop of mptc1 (amino acids 793-998), which normally binds the HH ligand, has been deleted in the Ptc1Δloop2 construct. Ptc1Δloop2 can thus maintain a constitutive blockade on SMO, acting
as a dominant negative regulator of HH signaling (Briscoe et al., 2001). The XEX-SHH expression vector was constructed to express cDNA encoding the 19.2 kD N-terminal fragment of SHH (Agarwala et al., 2001; Agarwala and Ragsdale, 2002).

**FOXA2**

Gain- and loss-of-function experiments for FOXA2 were carried out using FOXA2 (EFX-HNF3B or pMes-FOXA2-IRES-GFP), dnFOXA2 (pCAGGS-Fkh\(^{a2}\)-EnR–IRES-GFP) and FOXA2 RNAi (pSilencer-FOXA2-1663) containing expression vectors. EFX-HNF3B was constructed using the EcoRI fragment of mouse Hnf3b (pBSSK-mHnf3b) and ligating it into EFX3C (Sasaki and Hogan, 1993). pMes-FOXA2-IRES-GFP was created by subcloning the EcoRI fragment of EFX-HNF3B and ligating it into pMes-IRES-GFP. The pMes-IRES-GFP construct contains DNA encoding GFP downstream of the multi-clonal site region separated by an internal ribosomal entry site (IRES). This allows for the production of a single mRNA molecule that contains both FOXA2 and GFP coding regions. This strategy is useful in distinguishing cell autonomous effects as electroporated cells can be identified by the expression and detection of GFP. The construction of dnFOXA2 (pCAGGS-Fkh\(^{a2}\)-EnR–IRES-GFP) has been described previously (Jacob et al., 2007). The fusion of the Engrailed repressor domain (EnR) to FOXA2 acts to suppress transcription where FOXA2 normally binds to the DNA. The construction of pSilencer-FOXA2-1663 is detailed below under **Construction of shRNA expression vectors**.

**BMP Signaling**

Gain- and loss-of-function experiments for BMP signaling were carried out using EFX-Noggin, pXeX-dnBMPRI and EFX-BMP7. EFX-NOGIN is a secreted BMP antagonist and a gift from T. Shimogori (Shimogori et al., 2004). dnBMPRI is a dominant
negative form of BMP receptor I containing a K231R amino acid substitution (Zou et al., 1997). pXeX-dnBMPRI was constructed by ligating the SalI-BamHI fragment of dnBMPR1A with the expression vector pXeX (Johnson and Krieg, 1994). EFX-BMP7 was constructed by ligating the NotI fragment from pBSSK-mBMP7 (obtained from C. Tabin) with EFX3C.

**Nodal Signaling**

To test Nodal signaling, gain- and loss-of-function experiments were carried out using pCS2-Fast1<sup>SID</sup>, pCS2-Cyclops and pCS2-Smad2<sup>CA</sup>. pCS2-Fast1<sup>SID</sup> is a dominant negative form of Fast1 and acts to prevent the translocation of Smad2 to the nucleus which is required for the activation of the Nodal signaling pathway. pCS2-Cyclops is the zebrafish homolog of Nodal (Rebagliati et al., 1998). pCS2-Smad2<sup>CA</sup> is truncated at its N-terminus from amino acids 1-239 serving as a constitutively active form of Smad2, a downstream effector of the Nodal signaling pathway (Muller et al., 1999). Each of these expression vectors were obtained from U. Strahle (Muller et al., 2000).

**Plasmid constructs employed for in vitro transcription**

**EGFP**

The antisense riboprobe for EGFP was generated from pBS-EGFP, constructed by subcloning a BamH1-Not1 fragment containing the EGFP cDNA (pEGFPn1; Clontech) into the bluescript (pBS) plasmid (Stratagene).
BMP Signaling

*BMP7*, B. Houston (Houston et al., 1994); *CHORDIN*, A. Graham (Streit et al., 1998); *MSX1*, C. Tabin (Golden et al., 1999).

HH Signaling

*DISP1*, C. Ragsdale (Agarwala et al., 2005); *GLI2*, C. Tabin (Marigo et al., 1996a); *PTC1*, C. Tabin (Marigo et al., 1996b); *SHH*, C. Tabin (Riddle et al., 1993).

Notch-Delta Signaling

*NOTCH1*, J. Lewis (Myat et al., 1996); *SERRATE1*, J. Lewis (Myat et al., 1996).

Dorsal Markers

*GLI2*, C. Tabin (Marigo et al., 1996a); *MSX1*, C. Tabin (Golden et al., 1999); *PAX7*, M. Goulding (Goulding et al., 1993).

Midbrain-Hindbrain Boundary Markers

*FGF8*, G. Martin (Crossley et al., 1996); *GBX2*, M. Wassef (Niss and Leutz, 1998); *LMX1B*, C. Tabin (Riddle et al., 1995); *OTX2*, M. Wassef (Bally-Cuif et al., 1995); *WNT1*, A. McMahon (Hollyday et al., 1995).

Roof Plate Markers

*GDF7*, T. Jessell (Lee et al., 1998); *LMX1B*, C. Tabin (Riddle et al., 1995); *WNT1*, A. McMahon (Hollyday et al., 1995).
Floor Plate Markers

*FOXA2*, T. Jessell (Ruiz i Altaba et al., 1993a); *SHH*, C. Tabin (Riddle et al., 1993).

Medial Floor Plate Markers

*BMP7*, B. Houston (Houston et al., 1994); *CHORDIN*, A. Graham (Streit et al., 1998); *DISP1*, C. Ragsdale (Agarwala et al., 2005); *LMX1A; LMX1B*, C. Tabin (Riddle et al., 1995); *NGN2*, T. Jessell, (Novitch et al., 2001).

Ventral Midbrain Cell Fate Markers

*BRN3A*, S. Lamont (Heltemes et al., 1997); *EVX1*, G. Martin (Pierani et al., 1999); *ISL1*, T. Jessell (Tsuchida et al., 1994); *PAX6*, M. Goulding (Goulding et al., 1993); *PHOX2A*, C. Goridis (Ernsberger et al., 1995).

Dopaminergic Progenitor and Neuron Markers

*TH*, C. Ragsdale (Agarwala and Ragsdale, 2002); *PITX3*, C. Ragsdale (Garfin, 2004); *NURR1*, O. Conneely (Law et al., 1992); *LMX1A; LMX1B*, C. Tabin (Riddle et al., 1995); *FOXA2*, T. Jessell (Ruiz i Altaba et al., 1993a).

Cell Cycle and Differentiation Markers

*CLASS III BETA-TUBULIN*, C. Ragsdale (Sanders et al., 2002); *CYCLIN B2*, E. Nigg (Gallant and Nigg, 1992); *CYCLIN D1*, J. Lahti (Wianny et al., 1998).

Miscellaneous

Nodal, N-Cadherin, Shroom3
Construction of plasmids for in vitro transcription

In order to generate a plasmid from which we could produce an antisense riboprobe to a gene of interest, we subcloned PCR fragments from cDNA libraries into the pCRII TOPO vector (Invitrogen).

Primer Design

Primers were designed using MacVector (v7.1.1, Accelrys) under default settings to generate a 400-600 bp product from known or predicted cDNA sequences (NCBI). Primers were ordered from Integrated DNA Technologies and diluted to create a 100 μM stock solution. The following primers were used to generate PCR products:

**LMX1A**
Forward: 5’-ATGGACAGCGACGATACCTCAC-3’
Reverse: 5’-CCAGACCTACCTCCTGAAACAAGC-3’

**N-Cadherin**
Forward: 5’-TCAACAGCAACCGTGCTCCATTAC-3’
Reverse: 5’-ACATCATAGATACCAGCCTCCAGG-3’

**Nodal**
Forward: 5’-CACGCTCTGTCCTTTTCCCTG-3’
Reverse: 5’-GCTTTGTCCTTGAGAAGACGAG-3’

**Shroom3**
Forward: 5’-AAGCAACTCCAGCAGAATGCC-3’
Reverse: 5’-TTCCACAGACACTGATTTCCTCG-3’
Polymerase Chain Reaction

A 50 μL PCR reaction was set up using nuclease free water (Sigma), forward and reverse primers (100 nM final concentration), PCR Master mix (Eppendorf) and approximately 500 ng of an age and tissue specific cDNA pool (for details, see Generation of cDNA). All of the generated PCR products were run on a 1.5% agarose gel and compared to their predicted sizes. If the PCR product was of the predicted size the band was excised from the gel and the DNA was purified using a gel extraction kit (Qiagen). Alternatively, if the PCR reaction generated only a single band of the correct size, it could be directly used in the ligation reaction. Ligations using PCR reactions rather than gel purified bands were much more efficient.

Ligation and Transformation

The ligation and transformation was carried out according to the protocol provided by the TOPO TA Cloning kit (Invitrogen) with slight modifications. Modifications included using only 0.5 μL TOPO vector instead of 1.0 μL, 4.5 μL of PCR product if it was gel purified or 1 μL straight from the PCR reaction. 2 μL of the ligation reaction was used in the transformation of chemically competent cells, supplied with the kit. The transformed cells were transferred to ampicillin plates coated with X-gal (40 mg/mL in DMSO) and IPTG (100 mM in ddH₂O) for blue/white screening. White colonies were selected, grown up and sequenced with either Sp6 or T3 polymerases to confirm proper fragment ligation.

RNA isolation

Total RNA was isolated from E2 – E6 embryos using the RNeasy mini kit (Qiagen) with slight modifications. Embryos were harvested and if required,
subdissected, and placed into an RNA stabilization solution (RNA Later, Ambion). Approximately three E2 whole embryos or three E5-E6 midbrains yielded a sufficient amount of RNA. When finished, tissue was removed from RNA Later and placed into a 1.5 mL eppendorf tube containing 600 μL Buffer RLT and 6 μL β-mercaptoethanol. The tissue was then homogenized with a syringe and 31G needle, passing the lysate approximately 5 times. Total RNA was isolated from the lysate as per the protocol, eluting the final spin column with 20 μL RNAse free ddH₂O. The RNA concentration and purity was measured via NanoDrop (ND-1000, Thermo Scientific).

**Generation of cDNA**

cDNA pools were generated using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The reverse transcriptase reaction was carried out using 1 μg total RNA and oligo(dT)₂₀ primers, producing cDNA strictly from mRNA due to the presence of a poly-A tail. The purity and concentration of cDNA was measured via NanoDrop.

**Construction of shRNA expression vectors**

In order to knockdown target gene function, we used an expression vector-based system to generate short hairpin RNAs (shRNAs) that were specific to a complementary region of an mRNA of interest. This strategy is ideal for an in vivo system where the effect of gene silencing can be inherited to daughter cells through plasmid DNA. Once expressed in the cell, shRNAs are identified by Dicer, which cleaves the hairpin and
produces a siRNA molecule. The generated siRNA then binds to the RISC complex where together, they identify complementary regions in RNA molecules and cleave them into 21-22 nucleotide (nt) fragments, facilitating gene silencing.

**Identifying siRNA targets**

Optimal targets within an mRNA were identified by a proprietary algorithm (Ambion, http://www.ambion.com/techlib/misc/siRNA_finder.html). Full length mRNA sequences were submitted with the option to end siRNAs with a UU, a GC content not higher than 50%, and avoiding runs of four or more for any nucleotide. From the generated list, identified targets were blasted against the NCBI database to check for homology. Targets were selected only if they possessed mismatches of 4 or more nucleotides to the most homologous sequence. An siRNA generated with a mismatch of 4 or more to a selected target is ineffective in knocking down mRNA levels (Katahira and Nakamura, 2003). In general, between 2-4 targets and thus shRNA constructs were generated for each gene of interest as not all shRNAs effectively knocked down their targets. Additionally, selected targets were evenly distributed along the length of the mRNA.

**Construction**

Once a target was selected, DNA sense and antisense strands were generated encoding a 21 nt region of RNA complimentary to the target sequence in the mRNA of interest, a loop sequence of TTCAAGAGA, and a 21 nt region complimentary to that of the 5’ region enabling the formation of a hairpin. Additionally, the DNA sense and antisense strands were designed to include 4 nt overhangs for the recognition sites of EcoRI (AATT) and Apal (GGCC) at the 5’ and 3’ ends, respectively, for directional subcloning into the expression vector pSilencer (Ambion). pSilencer utilizes a U6 RNA
polymerase III promoter for RNA transcription and thus shRNA generation. The U6 promoter works much more efficiently in the chick than mammalian based CMV promoters (Das et al., 2006).

DNA oligos were ordered from Integrated DNA Technologies and diluted with ddH₂O to make a 1 µg/µL stock solution. The top and bottom strands were annealed in a 50 µL volume using 2 µL from each stock solution and 46 µL of annealing buffer (100mM KAc + 30 mM HEPES KOH + 2 mM MgAc in ddH₂O). The 50 µL solution was incubated at 90°C for 3 minutes and then for an hour at 37°C. The oligo mixture was then diluted to 8 ng/µL by adding 450 µL ddH₂O, which could either be used directly in ligation or stored at -20°C. 8 ng of annealed oligos were ligated to 100 ng of pSilencer, linearized with EcoRI and Apal, overnight at room temperature. The ligation reaction was used to transform competent cells, which were sequenced to confirm accurate construction.

**Primers used**

cFOXA2_478 top:  5’-
CACGTACATGACCATGCTCCTCAAGAGACGACATGGTCATGTACGTGTTTTTT-3’
cFOXA2_478 bottom:  5’-
AATTAAAAACACGTACATGACCATGCTCCTCTTTGAACGACATGGTCATGTACGTGGGCC-3’
cFOXA2_1663 top:  5’-
CTCCTCCTAAAGGCAAAGGTTCAAGAGACCTTTGCCTTTAGGAGGAGTTTTTT-3’
cFOXA2_1633 bottom:  5’-
AATTAAAAACTCCTCCTAAAGGCAAAGGTCTCTTTGAACCTTTTGCCCTTTAGGAGGAGGGGCC-3’
Riboprobe synthesis

To detect endogenous mRNA by in situ hybridization, antisense riboprobes were synthesized using digoxigenin (DIG)- or fluoroscein (FL)-labeled ribonucleotides (Roche Biochemicals) as per the manufacturer's directions employing T3, T7, or SP6 bacteriophage polymerases (Promega Inc.). DIG- and FL-labeled riboprobes were purified using standard LiCl/ethanol or ammonium acetate/isopropanol precipitation, respectively. Riboprobes were resuspended in RNAse free water and subsequently run on an agarose gel to evaluate the transcriptional efficiency and integrity. Riboprobes generated for in situ hybridizations could be stored up to one year at -20°C before use.

In situ hybridization

Fixation, Dehydration, and Rehydration Treatments

Embryos were harvested at the desired developmental stage and immersion-fixed in PFA, placed into a glass scintillation vial (RPI Corp.) and stored at 4°C. In addition, the neural tube of all embryos was opened to avoid trapping of reagents during antibody incubation and the color reaction. If required, further dissection of the embryos was carried out to remove the extraembryonic membranes, which ensured complete penetration of the reagents. Harvested tissue was fixed overnight at 4°C. Following dissection and overnight fixation, embryos were washed twice in PTw (1% Tween-20 (Sigma) in DEPC (Sigma)-treated PBS) and then dehydrated through increasing concentrations of methanol in PTw (25%, 50%, 75% and twice in 100% Methanol). Dehydrated embryos were stored in 100% Methanol at -20°C overnight. The following day, embryos were rehydrated through decreasing concentrations of methanol in PTw
(75%, 50%, and 25%), followed by two washes in PTw. Next, embryos were bleached in 6% H₂O₂ (Fisher Scientific) in PTw for one hour, followed by two short PTw washes. The embryos were then washed in a detergent cocktail solution (1% Nonidet P-40 (Calbiochem), 1% SDS (BioRad), 0.5% Deoxycholate (Calbiochem), 50 mM Tris-HCL (pH 7.5), 1 mM EDTA (pH 8.0), and 150 mM NaCl (Fisher Scientific)) three times for 30 minutes. This step sufficiently increases the permeability of the tissue to reagents used in the antibody detection and pigment deposition. The detergent washes were immediately followed by an additional fixation treatment (0.2% glutaraldehyde (Electron Microscopy Sciences), 1% Tween 20 in PFA) for 20 minutes. Two short PTw washes were then performed to remove the fixative. Afterwards, embryos were placed into a prehybridization solution (50% formamide (Fluka), 5x SSC (3 M NaCl, 0.3 M Na₃citrate•2H₂O and citric acid to pH 4.5), 1% SDS, 500 µg/ml tRNA from Brewer’s Yeast, 200 µg/ml acetylated BSA (Ambion), 50 µg/ml heparin sodium salt from porcine intestinal mucosa (Sigma)) and left to equilibrate to it (i.e., sink to the bottom of the vial) and stored at -20°C. Embryos could be stored in the prehybridization solution at -20°C for up to one year before use.

Hybridization

Embryos were incubated in prehybridization solution in a water bath at 72°C for at least one hour prior to the addition of antisense riboprobes. This period of incubation ensured that the majority of endogenous RNA was denatured and thus accessible to antisense riboprobes. RNA probe(s) were then added and distributed thoroughly throughout the prehybridization solution to obtain a 0.1-0.5 µg/ml probe concentration. Hybridization of the antisense riboprobe continued overnight for 16-20 hours at 72°C.
Post-hybridization Washes to Antibody Incubation

Following the hybridization reaction, embryos were washed three times for 30 minutes followed by an additional wash of 90 minutes in preheated Solution X (50% formamide, 2x SSC, 1% SDS) at 72°C. Embryos were transferred to 7 mL polycarbonate vials (Bellco Glass, Inc) and washed three times in TBST (TBS (25 mM Tris-HCl; pH 7.5, 136 mM NaCl, 2.68 mM KCl (Fisher Scientific)), 1% Tween 20). An incubation in 10% heat inactivated lamb serum (Invitrogen) in TBST at room temperature for two hours followed to prevent non-specific antibody binding. During the incubation in blocking solution, 1 \( \mu \)L of anti-digoxigenin or anti-fluorescein (Roche) antibodies conjugated to alkaline phosphatase were incubated with chicken embryo powder in 500 \( \mu \)L TBST in a 1.5 mL eppendorf tube, horizontally rocking at 4°C for one hour, to absorb any non-specific antibodies. The embryos were then incubated for 16-20 hours (max of 60 hours) at 4°C in TBST containing 1% lamb serum and the preabsorbed antibody (1:7500).

Post-Antibody Washes

After antibody incubation, the embryos were washed in TBST three times for 10 minutes, followed by five washes for 60 minutes. The embryos were then left overnight at room temperature.

First Color Reaction

To detect the hybridization of antisense riboprobes, embryos were washed in freshly prepared NTMT buffer (100 mM Tris-HCl at pH9.5, 100 mM NaCl, 50 mM MgCl\(_2\), 1% Tween 20) three times for 10 minutes. In a high pH solution, the alkaline phosphatase activity of the anti-DIG or anti-FL antibodies in combination with a phosphate substrate (175 \( \mu \)g/ml 5-bromo-4-chloro-indoxyl phosphate (BCIP, Roche,
100% DMF) and either nitro blue tetrazolium (NBT, Roche, 175 μg/ml, 70% DMF) or filtered (0.45 μm) tetranitro blue tetrazolium (500 μg/ml, TNBT, in 85% DMF) leads to a deposition of blue or brown chromagens, respectively. The tissue is incubated in NTMT with the proper amount of BCIP, and either NBT or TNBT in the dark at room temperature until the color reaction is completed. The duration of the color reaction varies among probes and is dependent upon the strength of expression of the mRNA of interest and the amount and integrity of the antisense riboprobe during hybridization. To stop the color reaction, three short washes in TBST and one overnight is sufficient for NBT deposition. In the case of TNBT, a 30 minute incubation in TE Buffer (10 mM Tris-HCl at pH 7.5 and 10 mM EDTA at pH 8.0), followed by three short washes in TBST and one overnight is necessary. After washing in TBST overnight, the tissue can be stored in NBF (10% formalin in PBS) at 4°C indefinitely.

In general, the best in situ hybridization results were achieved when the time between the initial tissue harvesting and the final color reaction was minimized. Additionally, the TNBT reaction worked best when it was made at least one day before the color reaction and when it was used within 14 days of suspension in H2O and DMF.

Two-Color in situ Hybridization

When the localization of two different mRNAs were to be analyzed in the same tissue, additional steps were required. First, both a DIG-labeled and FL-labeled antisense riboprobe were hybridized with tissue at the same time. After the completion of the first color reaction (either NBT or TNBT), the anti-DIG or anti-FL antibody needed to be stripped away as both possess alkaline phosphatase activity. This was achieved through three 30 minute washes in solution X at 72°C. Following the solution X washes,
tissue was processed as described above for antibody incubation, post-antibody incubation washes and the color reaction.

**Bromodeoxyuridine (BrdU) labeling and detection**

BrdU is a synthetic thymidine analog and incorporates into newly synthesized DNA. Thus, it can serve as a marker for cells that have recently undergone or are currently undergoing mitosis. 1 µl of BrdU (15 mg/ml in PBS; Sigma) was intravenously injected into E5 embryos electroporated at H&H 10. Injected embryos were placed back into the incubator for 30 minutes before they were harvested and immersion-fixed. Embryos were processed as per wholemounts for in situ hybridization with a few modifications. First, after the post hybridization washes, the tissue was washed three times in TBST followed by a 30 minute incubation of 2N HCl. The incubation with HCl serves to denature the chromosomal DNA in order to expose the BrdU antigen. Next, the tissue was washed three times for 10 minutes in pH 8.5 TBST in order to neutralize any residual acid. The tissue was blocked using 10% lamb serum at room temperature and then incubated for 2-3 days with an FL-conjugated anti-BrdU antibody (1:2000, Becton Dickinson). After the primary antibody incubation, three short washes followed by five one-hour washes and an extended wash overnight at 4°C in TBST was carried out. The detection of the FL antigen and color reaction was preformed as per in situ hybridization. When in situ hybridization was combined with BrdU labeling, the detection of the mRNA was carried out first as incubation of HCl causes the degradation of RNA.
Programmed cell death detection assay

In order to detect cells undergoing apoptosis, we utilized the ApopTag Peroxidase In Situ Apoptosis Detection kit (Chemicon International). During programmed cell death, DNA strands break and become fragmented. The TUNEL (terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-endlabeling) method labels the 3'-OH ends of fragmented DNA by incorporating nucleotides. Thus, when DIG-conjugated deoxynucleotides are provided and incorporated into fragmented DNA by TdT, cells undergoing apoptosis can be identified by immunohistochemistry.

Embryos at the appropriate time were harvested, subdissected, and immersion-fixed in PFA containing 0.1% Tween 20 for 3-4 hours at 4°C. They were dissected further if needed and subsequently dehydrated through a series of increasing ethanol concentrations in PBS (25%, 50%, 75% and twice in 100%) to be stored overnight at -20°C. The following day, the embryos were rehydrated into PBS through a decreasing ethanol series (75%, 50%, and 25%) at room temperature. The tissue was then permeabilized by three 30 minute washes in a detergent cocktail (as specified in the in situ hybridization protocol), followed by three five minute washes and one 30 minute wash in PTw. The embryos were washed in chilled equilibration buffer at 4°C. Next, the equilibration buffer was completely removed before adding just enough of chilled reaction buffer containing TdT to completely submerge the tissue. The enzymatic reaction was incubated at 4°C with gentle agitation overnight before being transferred to 37°C for two and one half hours. The reaction was terminated upon washing the tissue with Stop buffer at 37°C for 40 minutes. Three five minute TBST washes followed by five one hour washes were performed. The tissue was washed overnight in TBST at room temperature to ensure the removal of unincorporated nucleotides. The
endogenous phosphatase activity was inactivated through incubation at 65°C in TBST for 30 minutes. Subsequently the tissue was blocked in 10% lamb serum in TBST for two hours prior to overnight incubation in preabsorbed anti-digoxigenin alkaline phosphatase antibody (1:7500) and 2% lamb serum. Three five minute TBST washes, five one hour TBST washes, followed by an overnight TBST wash were performed to remove unbound antibody. To prepare the tissue for histochemical detection, embryos were washed in NTMT three times for 10 minutes. The alkaline phosphatase activity was detected through the use of 150 μg/ml NBT and 150 μg/ml BCIP in NTMT at room temperature. When the color reaction was completed, the tissue was rinsed three times and then washed overnight in TBST and stored in NBF.

**Section preparation**

**Gelatin embedding**

Fixed embryos were cryoprotected by equilibrating them to 20% sucrose and NBF at 4°C. Once the tissue was equilibrated (approximately 30 minutes), it was washed twice for 30 minutes in 20% sucrose in PBS, then equilibrated in 10% Porcine Gelatin 300 Bloom (Sigma) and 20% sucrose in PBS at 37°C for one hour. After one hour, the gelatin containing the tissue was poured into peel-a-way embedding molds (Polysciences, Inc.) and properly positioned before the gelatin solidified. The molds were then placed in an ice water mixture for one hour to ensure complete solidification. The gelatin was removed from the mold, trimmed and immersion fixed in a 20% sucrose and NBF solution at 4°C for at least four hours. The embedded tissue was then sectioned at the appropriate thickness (30-45 μm) on a freezing, sliding microtome (Leica). Sections were mounted onto Superfrost Plus slides (Fisher) and allowed to dry
overnight at 4°C. The mounted sections were dehydrated through a series of increasing ethanol concentrations (70%, twice in 95% and twice in 100%), cleared in Histoclear (National Diagnostics), and coverslipped with a permanent mounting media (Eukitt, Electron Microscopy Sciences).

**OCT embedding**

For immunostaining, embryos were collected, dissected, screened and immersion fixed in PFA for approximately 15-30 minutes. The tissue was cryoprotected by equilibration in 30% sucrose in PBS (approximately 30 minutes) at 4°C. The cryoprotected tissue was embedded and properly positioned in Tissue-Tek OCT compound (Sakura Finetek, Inc.) in a peel-a-way embedding mold. The tissue embedded in OCT was then frozen gradually either by floating in liquid nitrogen or in an ethanol dry ice mixture. The frozen block was trimmed and stored on dry ice or at -80°C. The embryos were sectioned on a microtome cryostat (Microm) at 12-20 μm and mounted on Superfrost Plus Slides. For best results, sections could be immediately processed for immunostaining or stored at -80°C until needed.

**Fluorescent labeling of sections**

**Immunostaining**

Slides containing sections were washed in PBT (0.1% Triton X (Sigma) in PBS) three times for 10 minutes. Next, sections were incubated in a blocking solution (5% heat inactivated goat serum (Invitrogen) and 2% BSA (Sigma) in PBT) for one hour at room temperature in a humidified chamber to prevent evaporation. Subsequently, primary antibody incubation occurred overnight at 4°C (2.5% goat serum and 1% BSA in
PBT) in a humidified chamber (concentrations for antibodies listed below). Prior to secondary antibody incubation (concentrations listed below), the tissue was washed three times for 10 minutes in PBT. Incubation of the secondary antibody was carried out at room temperature in a dark humidified chamber for one hour. The tissue was then washed three more times in PBT to remove excess secondary antibody. Slides were coverslipped using VectaShield mounting media (Vector Laboratories, Inc.) and sealed with clear nail polish.

**DAPI staining**

In order to label cell nuclei, sections were incubated with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen) at a concentration of 1:20,000 (of a 5 mg/ml stock) for 10 minutes in PBT. When DAPI staining was used in combination with immunostaining, DAPI staining was typically carried out last and only required a few short washes in PBT after the previous secondary antibody incubation was completed.

**Phalloidin staining**

Phalloidin-488 and 546 (Molecular Probes) are fluorophore-conjugated forms of a toxin that bind to and thus stain F-actin. In general, strong staining was observed after an incubation of only an hour (1:40 in PBT). Blocking is not required and when combined with antibody staining, only a few short washes in PBT after the use of phalloidin are necessary.

**Midbrain explants**

Midbrain explants were prepared by dissecting out presumptive midbrain from H&H 7-9 embryos in ice cold Leibovitz’s L-15 media containing glutamine (Gibco). In all
cases, the dorsal midbrain and the midbrain-hindbrain boundary were kept intact. After dissection, explants were transferred onto 30 mm diameter 0.4 μm culture plate inserts (Millipore) presoaked in Neurobasal media (Gibco) and positioned so that the pial surface contacted the membrane of the plate insert. The culture plate inserts were then placed into sterilized 6 well plates (Costar) containing 1 ml of Neurobasal media and incubated in a humidified 37°C CO₂ chamber for 1-3 days. The Neurobasal media was replaced every 24 hours. The tissue was immersion-fixed in PFA overnight at 4°C. RNA detection in midbrain explants was carried out exactly as described above for wholemounts.

**Cyclopamine treatment**

To block HH signaling early in the developing midbrain, explants were created by dissecting out presumptive midbrain from H&H 7-9 embryos with the dorsal midbrain and the midbrain-hindbrain boundary intact. For earlier explants, whole H&H stage 5 and 6 embryos were dissected out. Explants were either 1) exposed to 100 μM cyclopamine (Sigma) complexed with 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma) in Neurobasal media, or 2) HBC alone in Neurobasal media and cultured for 24 hours before harvesting according to previously published protocols (Incardona et al., 1998; Agarwala and Ragsdale, 2002; Norton et al., 2005).

**Whole-cell current-clamp recordings**

Embryos were explanted at H&H 10 as described above and neuronal progenitors visualized using infrared DIC microscopy (Zeiss Axioscope 2) and a Dage-
MTI Newvicon tube camera. Whole-cell current-clamp recordings were made at room temperature using somatic patch pipettes with open tip resistances of 2-4 MΩ. 30 μM Alexa 488 was added to the internal solution made according to published protocols (Scott et al., 2005). Dye-coupled cells were identified by visualizing Alexa 488 with fluorescence microscopy (EXFO X-cite 120 light source, Photometrics Cascade 512B camera).
Chapter 3: Regulation of Ventral Midbrain Patterning by Hedgehog Signaling

SUMMARY

In the developing ventral midbrain, the signaling molecule Sonic Hedgehog (SHH) is sufficient to specify a striped pattern of cell-fates (midbrain arcs). Here we asked whether and precisely how Hedgehog (HH) signaling might be necessary for ventral midbrain patterning. By blocking HH signaling by in ovo misexpression of Ptc1<sup>Δloop</sup>2, we show that HH signaling is necessary and can act directly at a distance to specify midbrain cell-fates. Ventral midbrain progenitors extinguish their dependence upon HH in a spatiotemporally complex manner, completing cell-fate specification at the periphery by Hamburger-Hamilton Stage 13. Thus, patterning at the lateral periphery of ventral midbrain is accomplished early, when the midbrain is small and the HH signal needs to travel relatively short distances (~30 cell diameters). Interestingly, single cell injections demonstrate that patterning in the midbrain occurs within the context of cortex-like radial columns of cells that can share HH blockade and are cytoplasmically connected by gap junctions. HH blockade results in increased cell-scatter disrupting the spatial coherence of the midbrain arc pattern. Finally, HH signaling is required for the integrity and the signaling properties of midbrain’s boundaries (e.g. the midbrain-hindbrain boundary, the dorsoventral boundary), its perturbations resulting in abnormal cell mixing across “leaky” borders.
INTRODUCTION

The developmental organization of any tissue requires the coordination of signals which emanate from specialized signaling centers located at tissue boundaries (Rubenstein et al., 1994). In the case of the midbrain, the identity of the ventral midbrain or rostral floor plate (rFP) as a signaling center is firmly established (Agarwala et al., 2001; Fedtsova and Turner, 2001; Blaess et al., 2006). The rFP occupies the ventral midline of the midbrain and secretes the signaling molecule, Sonic Hedgehog (SHH), whose role in pattern formation is the focus of intense study (Ingham and McMahon, 2001).

Hedgehog (HH) signal transduction begins with HH binding to its receptor and negative regulator, PTC1 (Marigo and Tabin, 1996; Stone et al., 1996; Ingham and McMahon, 2001; Hooper and Scott, 2005). In the absence of HH signaling, PTC1 maintains a constitutive block on the transmembrane protein, Smoothened (SMO) so that no signaling can occur (Alcedo et al., 1996; Akiyama et al., 1997). New findings suggest that in the absence of the ligand, PTC1 can induce provitamin D3 which binds SMO in adjacent cells to block HH activation (Bijlsma et al., 2006). In the presence of HH, the PTC1-mediated block on SMO is lifted. HH signaling then occurs through a complex cascade which eventually converges upon the activator- (GLI1, GLI2, GLI3) or repressor- (chiefly GLI3) function of the GLI/Ci family of transcription factors (Aza-Blanc et al., 1997; Dai et al., 1999; Sasaki et al., 1999; Litingtung and Chiang, 2000; Wijgerde et al., 2002; Bai et al., 2004).

Amongst vertebrates, one of the best understood examples of HH’s role in patterning is the ventral spinal cord (Jessell, 2000). Gain and loss of function studies
have shown that HH is both necessary and sufficient for cell-fate specification in the spinal cord (Chiang et al., 1996; Briscoe and Ericson, 2001; Zhang et al., 2001a). HH is required directly for cell fate specification and can pattern cell-fates at long range (~15-20 cell diameters) (Briscoe et al., 2001; Wijgerde et al., 2002).

A role for HH signaling in the regulation of cell affinities has been found in the fly wing imaginal disc and abdominal ectoderm (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence et al., 1999). In each tissue, differential HH signaling creates two compartments which display distinct and inheritable affinities. Thus, cells of a compartment and their lineal relatives cohere with each other and do not intermix with those of the other compartment. As a result, the compartments become separated by a sharp, lineage restriction boundary exhibiting signaling properties (Garcia-Bellido et al., 1973; Morata and Lawrence, 1975; Blair, 1992; Lawrence et al., 1999). These results implicate HH signaling in the establishment of tissue boundaries and in the maintenance of a spatially coherent pattern (Dahmann and Basler, 1999). A loss of spatial organization has also been reported in several HH pathway mouse (Shh;Gli3−/−; Smo;Gli3−/−; Gli2;Gli3−/−) and chick mutants (e.g. the talpid2) (Litingtung and Chiang, 2000; Wijgerde et al., 2002; Bai et al., 2004; Agarwala et al., 2005). Recently, HH signaling has also been implicated in the maintenance of orthogonal signaling centers in the vertebrate limb and the MHB of the neural tube (Aoto et al., 2002; Khokha et al., 2003; Blaess et al., 2006). However, whether regulation of boundaries is a general feature of HH action among vertebrates, is not yet known.

In this study, we have analyzed the role of HH signaling in the chick midbrain where stripes of cell-fates (midbrain arcs) develop parallel to the rFP source of SHH (Agarwala et al., 2001; Sanders et al., 2002). In vivo misexpression studies have shown
that ectopic SHH can recapitulate the entire midbrain pattern of cell-fates in a concentration-dependent manner (Agarwala et al., 2001; Agarwala and Ragsdale, 2002). No ventral cell-fates remain in the Shh\(^{-/-}\) mouse midbrain by E11.5 when the entire midbrain exhibits a dorsal phenotype (Fedtsova and Turner, 2001; Blaess et al., 2006). Although these studies demonstrate the importance of SHH in the developing midbrain, they do not permit a precise cellular and molecular analysis of the role of HH signaling in establishing midbrain pattern. Nor do they elucidate the physical nature of the HH signal, for example, its range (short or long), mode (direct or indirect), timing or duration of action.

To address these issues, we perturbed HH function in the ventral midbrain by in vivo misexpression of Ptc1\(^{\Delta}\text{loop2}\), a mutated form of PTC1, previously used to successfully block HH signaling (Briscoe et al., 2001; Kiecker and Lumsden, 2004). We show that HH is directly required for cell-fate specification within columns of midbrain cells which are cytoplasmically connected and likely to be clonally related (Noctor et al., 2001). HH signaling acts at long range (~31 cell diameters) at Hamburger and Hamilton (H&H) stage 13, when cell-fate specification is complete at the lateral periphery of ventral midbrain (Hamburger and Hamilton, 1951). Beyond this time, continued HH-dependence is only seen within lateral regions of the rFP and cell-fates associated with it. Our results also suggest that blockade of HH signaling increases cell-proliferation and inhibits differentiation within the midbrain. Finally, HH is required for the spatial organization of midbrain cell types and for the maintenance of the midbrain’s boundaries. Perturbations of HH signaling thus result in the admixture of midbrain cells with each other and with cells from juxtaposed tissues.
RESULTS

HH signaling is necessary for cell-fate specification in the ventral midbrain

The ventral midbrain pattern is composed of a set of arcuate territories arrayed parallel to the midline (rostral floor plate, rFP) source of SHH (Sanders et al., 2002). These are marked by the gene expression of PHOX2A and TYROSINE HYDROXYLASE (TH) in the most medial arc (arc 1), and more laterally (at a distance from the SHH source) by the expression of NKX2.2, PAX6 and EVX1 (Fig. 3.1A,F,G; data not shown; Agarwala and Ragsdale, 2002). We determined that the SHH source and the midbrain arc pattern were not perturbed by control electroporations of EGFP (Fig. 3.1A). The FP markers, SHH and FOXA2/HNF3ß are transcriptional targets of HH signaling in the midbrain (Agarwala et al., 2001) and were suppressed by Ptc1∆loop electroporations (Fig. 3.1B; Fig. 3.4D). Ptc1∆loop misexpression also prevented the correct specification of all ventral midbrain cell-fates, resulting in their respecification to more dorsal (e.g. PAX7+) fates (Fig. 3.1C; Fig. 3.4G,H; Fig. 3.6A). We noted a suppression of the PHOX2A+ neurons of the oculomotor complex, midbrain dopaminergic (TH+) neurons, as well as the territories (NKX2.2+, PAX6+, EVX1+) specified at a distance from the SHH source (Fig. 3.1D-G; data not shown). Taken together with our previous work, these results suggest that HH signaling is both necessary and sufficient for cell-fate specification in the ventral midbrain and can act directly at a distance to specify midbrain cell fates (Agarwala et al., 2001; Agarwala and Ragsdale, 2002).
Figure 3.1. HH signaling is necessary for cell-fate specification in the ventral midbrain

(A). EGFP-electroporated (right side) controls do not show disruptions in rostral floor plate (rFP, SHH+, brown) or in midbrain arc pattern formation at E5. Midbrain arcs are marked by the homeobox (HX, blue) gene expression of PHOX2A in the first arc (1), PAX6 (P6) and EVX1 (E1). (B). Blockade of FOXA2 (brown) expression following unilateral Ptc1Δloop2 (blue) electroporation. (C). Respecification of ventral cell-fates (marked by HX genes, blue) into dorsal (PAX7+, brown, arrowhead) cell-fates. (D, E). Blockade and bidirectional spread of PHOX2A+ (brown) oculomotor complex neurons following bilateral electroporation (E) of Ptc1Δloop2 (blue) compared to EGFP-electroporated controls (D). D and E are photographed at the same magnification. Note the lack (caudally, arrowhead) of overlap between PHOX2A and Ptc1Δloop2 transgene expression in E. Rostral cells (arrow) have extinguished their requirement for HH signaling by this stage (see text). (F, G). Reduced expression and spread of TYROSINE HYDROXYLASE (F, dopaminergic neurons), PAX6 (G, brown) and EVX1 (G, blue) following unilateral HH blockade. (H). Cross-section demonstrating the non-autonomous spread of PAX6+ (brown, arrowhead) cells following unilateral Ptc1Δloop2 (blue) electroporation. Note presence of ectopic PAX6+/ Ptc1Δloop2+ cells (arrow, see text). (I, J). E8 wholemounts electroporated at H&H10 demonstrating that compared to EGFP controls (I), cell spread following HH blockade (J) increases with time (compare with E5 brains in D and E) and is multidirectional. Blue: TH; brown: ISL1+ motor neurons. Abbreviations: 1: first arc; 2: arc 2; iii: third ventricle; bi: bilateral electroporation; E1: EVX1; EP: electroporated; P6: PAX6; HX: Homeobox expression of PHOX2A, PAX6, EVX1; MHB: midbrain-hindbrain boundary; rFP: rostral floor plate; TH: tyrosine hydroxylase; tec, tectum.
HH blockade results in cell spread and a disrupted midbrain arc pattern

In the fly wing and abdomen, perturbations of HH signaling result in abnormal cell movements due to altered cell-adhesivities (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence et al., 1999). Increased cell-spread was also noted within the ventral midbrain following Ptc1\(^{\Delta}\)loop2 electroporations (compare Fig. 3.1D and E; Fig. 3.E-J). This increased scatter was non-autonomous (e.g. Fig. 3.1E,H), multidirectional, increased dramatically over time (Fig. 3.1I,J, compare with Fig. 3.1D,E) and affected progenitors as well as differentiated neurons (Supplementary Fig. 3.7A). As a result of this scatter, a spatially coherent midbrain arc pattern could not be formed following Ptc1\(^{\Delta}\)loop2 electroporations (Blair and Ralston, 1997; Lawrence et al., 1999; Wijgerde et al., 2002; Bai et al., 2004).

HH signaling inhibits proliferation and induces neuronal differentiation in the midbrain

HH signaling is known to accelerate progression through the cell cycle in many model systems (Kenney and Rowitch, 2000; Duman-Scheel et al., 2002; Roy and Ingham, 2002). By contrast, we found that known cell cycle targets of HH signaling, CYCLIN B2, a marker of G2/M transition and CYCLIN D1, a marker of G1/S transition, and BrdU labeling (marking the S phase of the cell-cycle) all indicated greatly increased numbers of neuronal progenitors following Ptc1\(^{\Delta}\)loop2 electroporation (Fig. 3.2A-D; Masai et al., 2005). Concomitant to increased proliferation was a reduction in the number of
Figure 3.2. HH blockade prevents differentiation and promotes proliferation in the ventral midbrain

(A, B). Increased CYCLIN B2 (A) and CYCLIN D1 expression (B) following unilateral misexpression of Ptc1Δloop2. (C). BrdU labeling shown in cross-section through an EGFP-electroporated embryo where it is confined to proliferating cells of the ventricular layer. (D). Massive increase in BrdU labeling (blue) following Ptc1Δloop2 electroporation. Note that the increased thickness of the ventricular layer is associated with a reduction of the mantle layer where differentiated neurons normally reside (compare double headed arrows in C and D, photographed at the same magnification). (E). Cross-section through ventral midbrain showing a reduction in CLASS III β-TUBULIN expression (brown, asterisk) following HH blockade (E, Inset). Wholemount view of the section in E. (F, G). SHH (brown) overexpression results in reduced CYCLIN D1 (blue) expression. The same embryo is presented in G (before) and H (after) the detection of SHH. (H). Embryos bilaterally electroporated with either SHH (light embryos) or Ptc1Δloop2 (dark embryos) at H&H 9. Note the reduced size of SHH-electroporated embryos compared to Ptc1Δloop2-electroporated embryos. Embryos are shown in sagittal view, with rostral to the left. Abbreviations: III: third ventricle; bi: bilateral electroporation; Di: diencephalon; EP: electroporated; HB: hindbrain; rFP: rostral floor plate.
differentiated neurons demonstrated by the reduced thickness of the mantle layer (double-headed arrow, Fig. 3.2C,D) and reduced CLASS III β-TUBULIN expression (Fig. 3.2E,E inset). TUNEL labeling indicated no significant differences in cell death between Ptc1Δloop2 and EGFP-electroporated midbrains (Supplementary Fig. 3.7B, C).

To discount the possibility that the altered midbrain proliferation and differentiation is due to a peculiarity of the Ptc1Δloop2 construct itself, we misexpressed SHH and found that CYCLIN D1 was severely reduced in both ventral and dorsal midbrain (Fig. 3.2F,G; 3.8D; Guerrero and Ruiz i Altaba, 2003; Thibert et al., 2003). Finally, we compared the total size of midbrains electroporated at H&H 9 with either SHH or Ptc1Δloop2 and found that the SHH, but not the Ptc1Δloop2 electroporated midbrains displayed a massive (>50%, in some cases) reduction in size (Fig. 3.2H). Taken together, these results are consistent with a role for HH signaling in the midbrain in suppressing proliferation and inducing differentiation (Wijgerde et al., 2002; Bai et al., 2004; Masai et al., 2005).

**HH blockade reveals a cortex-like radial organization of the ventral midbrain neurepithelium**

Following HH blockade, the expression of appropriate midbrain cell-fates (e.g. FOXA2, PHOX2A) was not only blocked cell-autonomously within cells expressing the Ptc1Δloop2 (mptc1) transgene, but also in “haloes” immediately surrounding the Ptc1Δloop2+ cells (Fig. 3.3A; Fig. 3.1B,E). In E5 cross-sections, these “haloes” (cells which did not express spatially appropriate HH-target fates despite appearing Ptc1Δloop2 negative), were organized into “columns” of cells that spanned the ventricular-pial (radial) axis and were radially aligned with pially located Ptc1Δloop2+ cells (Fig. 3.3B).

This columnar organization has not yet been described in the midbrain. However,
it bore a remarkable resemblance to the neocortex, where cortical columns emerge to form a lineal relationship between neuronal precursors (radial glia) and their descendants as they colonize the cortical plate along the radial axis (Chenn and McConnell, 1995; Kriegstein and Noctor, 2004).

To determine whether midbrain columns were the result of HH blockade or a normal feature of midbrain organization, we shifted our analysis to E4 when the midbrain neurepithelium is predominantly composed of undifferentiated precursors and HH-blockade-mediated perturbation of proliferation and differentiation does not add additional complexity (Fig. 3.2A-E).

Columns of electroporated cells spanning the ventricular-pial axis could be seen in Ptc1\textsuperscript{△loop2}\textsuperscript{-}electroporated embryos at E4 (Fig. 3.3C). A similar columnar organization was seen in midbrains electroporated with low concentrations of EGFP (0.2 µg/µl) to yield only a few isolated EGFP+ cells per brain (Fig. 3.3D,E). Thus, HH blockade neither induced nor disrupted the columnar organization of the ventral midbrain. Notably, the EGFP+ cells displayed the characteristic morphology of radial glial/neuronal precursors (bipolar cells spanning the midbrain ventricular-axis and exhibiting apical and basal processes with end-feet) (Fig. 3.3E; data not shown; Noctor et al., 2001; Malatesta et al., 2003). Furthermore, when multiple EGFP+ cells were present within a single midbrain column, they appeared cytoplasmically connected (arrowhead, Fig. 3.3D). Cytoplasmic connections (via gap junctions) amongst clonal relatives are a feature of cortical columns and have been detected in dye-coupling experiments (Noctor et al., 2001). Indeed, single cell injections in midbrain explants at H&H 10 with Alexa 488 (which crosses gap junctions, but does not diffuse across cell membranes) resulted in the instantaneous labeling of up to 3 cells, demonstrating the presence of gap junctions among midbrain
progenitors (n=5; Fig. 3.3F).

A detailed description of midbrain columns will be published elsewhere (Bayly and Agarwala, unpublished observations). We propose that columns of Ptc1\(^{\Delta}\text{loop2}-\) cells radially associated with the Ptc1\(^{\Delta}\text{loop2}+\) cells are unable to express appropriate HH-target fates because they divide and differentiate under reduced HH conditions. Such conditions could be created by the cytoplasmic inheritance of low/undetectable levels of Ptc1\(^{\Delta}\text{loop2}\) (cell-autonomous) or due to the transfer of small inhibitory molecules (e.g., provitamin D3) among neuronal precursors via gap junctions (Bijlsma et al., 2006). For precision, we have described the radial effects of Ptc1\(^{\Delta}\text{loop2}\) electroporations as being “radially associated” or “associated” with Ptc1\(^{\Delta}\text{loop2}+\) cells, rather than being cell-autonomous or non-autonomous.

**Spatiotemporal regulation of ventral midbrain patterning by HH**

We next determined the spatiotemporal sequence in which midbrain cell-fates extinguished their dependence upon HH signaling. Compared to EGFP-electroporated controls (Fig. 3.4A, Fig. 3.5A), very few Ptc1\(^{\Delta}\text{loop2}+\) cells were seen within the medial region of rFP in bilateral electroporations (Fig. 3.4B,F; Fig. 3.5B; Briscoe et al., 2001; Wijgerde et al., 2002). When they did appear at the midline, they could only suppress SHH or FOXA2 gene expression along the caudal (near the MHB), but not anterior midline (arrowhead, Fig. 3.4B) of rFP between H&H 6-11 (Fig. 3.4B,C; Fig. 3.8A). In sharp contrast, FP cell-fates (SHH, FOXA2) could be blocked in lateral regions of rFP in electroporations conducted between H&H 15-20 (Fig. 3.4D; Fig. 3.8A-C).
Figure 3.3. **Ptc1Δloop2 affects cell-fate specification in a radial manner**

(A). “Haloes” of PHOX2A/-/Ptc1Δloop2- cells surround Ptc1Δloop2+/ PHOX2A-(blue) cells. (B). Cross-section through A demonstrating that the “haloes” are columns of PHOX2A-/Ptc1Δloop2- cells, radially associated with more pially located Ptc1Δloop2+ cells. (C). Ptc1Δloop2 electroporated embryos at E4 display midbrain columns in cross-section. (C, Inset). A magnified view of a single column of cells indicated by arrowhead in C. Individual cells are marked by asterisks. (D). Cross-section through an E4 embryo electroporated with low concentrations of EGFP displaying bipolar-radial glia-like midbrain progenitors. Note that when multiple cells are present in a single column, they are cytoplasmically continuous (arrowhead). (E). Close-up of boxed area in D, highlighting the radial glial-like morphology of midbrain progenitors, including the presence of end-feet at the ventricular surface. (F). Demonstration of dye-coupling via gap junctions among 3 ventral midbrain cells following the injection of Alexa 488 into the central cell (*) in an H&H 10 explant presented in a wholemount view (rostral is to the top and ventricular surface faces the viewer. Orientation is the same as in 1A). Each cell is ~7.5 µm across and the cells are spaced ~5 µm apart. The central cell (*) is ventricular with respect to the other 2 cells. **Abbreviations:** rFP: rostral floor plate.
In contrast to the rFP, Ptc1Δloop2 misexpression between H&H 8-13 (n>40) resulted in the uniform blockade of all arc-specific cell-fates throughout the ML (mediolateral) axis of ventral midbrain (Fig. 3.4E,F). Although, ML differences in specification were not noted across the midbrain arcs following HH blockade during this time, cell-fate specification was more severely affected near the MHB compared to more rostral regions, particularly within the medial arc territory (7/10 embryos; Fig. 3.4F). In electroporations beyond H&H 13, only cell-fates associated with the lateral regions of rFP and arc 2 (the region between the PHOX2A and PAX6 territories) were affected by HH blockade (Fig. 3.4G,H; 3.8E). Intriguingly, this region was marked by the ectopic presence of dorsal phenotypes (e.g. PAX6+) occurring both non-autonomously (arrowhead, Fig. 3.4H) and in radial association with misexpressed Ptc1Δloop2 (arrow, Fig. 3.4H). We saw a similar mixed phenotype (radially associated and non-autonomous) throughout the study and interpret these results as a combination of respecified cell-fates (to a more dorsal identity, radially associated with Ptc1Δloop2+ cells) and abnormal cell-scatter (non-autonomous; See Discussion).

Our data suggest that anterior midline rFP was not affected in our manipulations between (H&H 6-20) and may be specified earlier or independent of HH signaling (Patten et al., 2003). HH-mediated specification of the remaining ventral midbrain cell fates occurs in at least 3 temporal phases (Fig. 3.8A-C). The caudo-medial region of rFP becomes independent of HH signaling first, prior to H&H 11 (Step 1; Fig. 3.8A), followed by most ventral midbrain cell fates by H&H 13 (Step 2, Fig. 3.8B). Beyond H&H 13, only the lateral regions of rFP and cells associated with it exhibit a dependence upon HH signaling and continue to do so at least until H&H 17-20 (Step 3; Fig. 3.8C).
Figure 3.4. Spatiotemporal regulation of HH requirement in the ventral midbrain (A). Bilateral EGFP (blue) misexpression does not perturb rFP expression (FOXA2, brown). (B, C). Caudal-medial and lateral, but not antero-medial regions (arrowhead, B, C) of rFP (FOXA2+, brown) can be disrupted following bilateral electroporation of Ptc1Δloop2 (blue) at H&H 6-9. C is a cross-section of B at the level indicated by the line in B. Note the meager number of Ptc1Δloop2+ cells at the midline (arrowheads, B, C) compared to controls (A). (D). HH blockade disrupts lateral rFP specification at H&H 15-16. (E). E6 Embryo electroporated with Ptc1Δloop2 (blue) between H&H 9-11 demonstrating the uniform blockade of cell-fate specification in all midbrain arcs, assayed by HX gene expression (brown). Note the extensive cell mixing and disruption of the arc pattern. (F). Greater caudal perturbation of the PHOX2A+ (1, brown, arrowhead) first arc following Ptc1Δloop2 electroporation (blue) at H&H 10-12. The rostral expression of PHOX2A (arrow) is largely unaffected despite the higher bilateral expression of the Ptc1Δloop2 transgene in this region. (G). E6 embryo electroporated between H&H 17-20 demonstrating that midbrain cell-fates (brown) are independent of HH signaling except in lateral regions of the rFP and cells associated with it (e.g. arc 2). (H). Close up of boxed area in G demonstrating that midbrain progenitors within the lateral region of rFP and cells associated with it (e.g. arc 2) can be respecified to more dorsal (PAX6+) cell fates in association with Ptc1Δloop2+ cells (arrow) and dorsal cells (PAX6+) can move into this region non-autonomously (arrowhead). Abbreviations: 1: first arc; 2: arc 2; III: third ventricle; bi: bilateral electroporation; E1: EVX1; EP: electroporated; P6: PAX6; HX: Homeobox expression of PHOX2A, PAX6, EVX1; MHB: midbrain-hindbrain boundary; rFP: rostral floor plate.
Perturbations of HH signaling result in a disruption of midbrain boundaries

In the fly wing and abdomen, HH perturbations result in a disruption of cell affinities, manifest as a spatially disorganized pattern and disrupted compartment boundaries (Fig. 3.1; Lawrence et al., 1999). We asked if midbrain boundary perturbation accompanied the disruption of spatial pattern as well (Lawrence et al., 1999; Aoto et al., 2002; Zervas et al., 2005; Blaess et al., 2006).

The Midbrain-Hindbrain Boundary (MHB)

\( \text{Ptc1}^{\text{\Delta loop2}} \) misexpression resulted in a broadening of the MHB and a non-autonomous scattering of \( \text{WNT1}^{+} \) cells not seen in the control brains (Fig. 3.5A,B). Strikingly, \( \text{Ptc1}^{\text{\Delta loop2}} \) manipulations resulted in the intermingling of midbrain (\( \text{OTX2}^{+} \)) and MHB/hindbrain cells (\( \text{FGF8}^{+} \); Fig. 3.5C,D; 3.8F). This was accompanied by a dramatic broadening of the \( \text{FGF8}^{+} \) MHB territory (Fig. 3.5C,D). The broadening could not be explained by a repression of \( \text{OTX2} \), an expansion of \( \text{GBX2} \) or the ectopic presence of mis-specified cells (Fig. 3.5D, Supplementary Fig. 3.7F). Instead, the MHB broadening could be attributed to enhanced cell proliferation within the MHB as demonstrated by the dramatic increase in of \( \text{CYCLIN D1}^{+}/\text{FGF8}^{+} \) cells (Fig. 3.5E,F). Thus, reduced HH signaling results in an enlarged MHB which is not sharply defined and across which cell-mixing can occur (Vaage, 1969; Zervas et al., 2004).

The Dorsoventral (DV) Boundary

The disruption of the MHB following \( \text{Ptc1}^{\text{\Delta loop2}} \) manipulations prompted us to examine the DV boundary. When electroporated with \( \text{Ptc1}^{\text{\Delta loop2}} \), ectopic \( \text{PAX7}^{+} \) cells,
Figure 3.5. Disruption and cell-mixing at the chick MHB following HH blockade
(A, B). Unlike controls (A), bilateral electroporation of Ptc1Δloop2 (blue) disrupts WNT1
(brown, arrowhead) expression at the MHB. (C, D). Unlike controls (C), HH blockade
(D) results in the broadening of FGF8 expression at the MHB (blue; compare the length
of the double headed arrows in C and D). Note the ectopic mixing of FGF8+ (white
arrowhead) and OTX2+ (brown, black arrowhead) (D). C and D were photographed at
the same magnification. (E, F). Increased CYCLIN D1 expression within the MHB
following bilateral Ptc1Δloop2 electroporations. E and F are photographs of the same
embryo demonstrating that all FGF8+ cells (arrows, brown) are also CYCLIN D1+. However,
all ectopic CYCLIN D1+ (arrowheads) cells are not FGF8+ (See Fig. 3.2A, left
side for normal CYCLIN D1 expression). Abbreviations: III: third ventricle; bi: bilateral
electroporation; EP: electroporated; HB: hindbrain; MHB: midbrain-hindbrain boundary;
rFP: rostral floor plate.
normally confined to dorsal midbrain, were noted in the ventral midbrain (Fig. 3.6A). We also observed that the expression of the DELTA homolog, SERRATE1, was disrupted along the DV boundary following Ptc1-loop2 electroporations (Fig. 3.6B). The presence of PAX7+ cells in ventral midbrain could result from a conversion of ventral midbrain cells to a dorsal fate or from the movement of dorsal cells into ventral midbrain because of a breach in the signals that normally restrict their admixture. To distinguish between these possibilities, we resorted to an explant system, where all PAX7+ dorsal tissue could be removed prior to electroporation with Ptc1-loop2 (Agarwala and Ragsdale, 2002). In EGFP-electroporated control explants with or without an intact tectum, no PAX7+ cells were ever seen in ventral midbrain (n=11/11; Fig. 3.6C; data not shown). When explants prepared without any associated PAX7+ tissue (dorsal midbrain and hindbrain; n=4/4) were electroporated with Ptc1-loop2, PAX7+ cells could be observed within the ventral midbrain, suggesting that some ventral midbrain cells were converted to a dorsal (PAX7+) phenotype in the absence of HH signaling (Fig. 3.6D).

In the absence of any tectum or dorsal hindbrain, the in vitro experiments presented in Fig. 3.6D cannot definitively rule out the additional possibility of movement of cells from adjacent tissues as noted before (Fig. 3.4G,H; Fig. 3.5A-D). To resolve this, we resorted to in vivo misexpression of Ptc1-loop2 near the DV boundary followed by the simultaneous detection of PAX7 and Ptc1-loop2 transgene. Ectopic PAX7+ cells were not seen in EGFP-electroporated brains near the DV boundary (n=0/5; Fig. 3.6E). However, there was always a small number of cells that displayed PAX7 expression non-autonomously in Ptc1-loop2 electroporated brains (n=7/7; Fig. 3.6F). Taken together, our results are consistent with both a transformation of ventral midbrain fates to
Figure 3.6. HH blockade leads to a disruption of the DV boundary

(A). Ectopic PAX7 in ventral midbrain after HH blockade. (B). SERRATE1 expression (blue), normally confined to dorsal midbrain (tec) and to a thickening at the DV boundary (arrowhead), is perturbed in Ptc1Δloop2 electroporations. (C). Absence of PAX7+ (blue) cells in the ventral midbrain of EGFP (brown) electroporated explants. Note the presence of PAX7 (blue) expression in the tectum (tec). (D). Bilateral Ptc1Δloop2 electroporation induces ectopic PAX7 in ventral midbrain explants with no associated tectal tissue. (E, F). EGFP misexpression (blue) near the D/V boundary (dashed line) fails to perturb PAX7 expression (brown) in (E). In (F), Ptc1Δloop2 misexpression (blue) near the D/V boundary (dashed line) induces ectopic PAX7+ (brown) cells, some non-autonomously (arrowhead). Arrow points to the upregulation of PAX7 in association with Ptc1Δloop2 misexpression. Abbreviations: III: third ventricle; bi: bilateral electroporation; EP: electroporated; HB: hindbrain; rFP: rostral floor plate; tec: tectum.
dorsal fates and a non-autonomous movement of dorsal cells into ventral midbrain due to an MHB-like disruption of the DV boundary.
Figure 3.7. Supplementary Figures

(A). Unilateral HH blockade results in the spread of PHOX2A+ precursors and differentiated neurons in a cross-section through an E6 midbrain. Dashed line delineates the ventricular-mantle layer boundary. (B, C). TUNEL labeling at E5 demonstrating a comparable increase in cell death on the electroporated (right) side after unilateral EGFP (S2) and Ptc1^Δloop2 (S3) electroporations. (D). Unilateral misexpression of SHH results in reduced CYCLIND1 expression (*) in tectum. (E). Cross-section through the brain shown in Fig. 3.4G and H demonstrating that HH blockade after H&H 13 does not affect the specification of lateral (EVX1+) cell fates. (F). HH blockade results in mixing of OTX2+ (blue, arrowhead) and hindbrain cells (arrowhead). Although GBX2 (blue) expression is present in lateral hindbrain, it is absent from the hindbrain ventral midline where cells are electroporated and ectopic OTX2 is seen.
DISCUSSION

In this study we focused on the cellular and molecular mechanisms governed by HH signaling in the ventral midbrain and summarize our conclusions in Fig. 3.8. We show that HH acts within columns of cytoplasmically connected midbrain progenitors to directly specify cell-fates at a distance (Fig. 3.8E; Kriegstein and Noctor, 2004). The specification of HH-target midbrain cell-fates is largely complete by H&H 13 with a continued requirement for HH signaling beyond this time point only in lateral regions of rFP and associated cell-types (e.g. arc 2; Fig. 3.8A-C). Interestingly, Ptc1\textsuperscript{loop2} electroporations result in increased cell proliferation and reduced differentiation, closely resembling size regulation in the Gli2:Gli3\textsuperscript{-/-} and Smo:Gli3\textsuperscript{-/-}, but not the Shh\textsuperscript{-/-} mice (Fig. 3.8E; Litingtung and Chiang, 2000; Wijgerde et al., 2002; Bai et al., 2004). Finally, HH signaling is required for the correct spatial patterning of midbrain cell-types and for the integrity of midbrain’s boundaries (MHB, DV boundary; Fig. 3.8D).

The range of HH action in the midbrain

We determined that direct HH signaling was required at the lateral edge of ventral midbrain and that this requirement was extinguished by H&H 13 (Figs 3.1, 3.4). The restriction of PAX7 to dorsal midbrain by HH is a measure of the range of HH signaling (Ericson et al., 1996; Wijgerde et al., 2002). The distance between the lateral limit of the SHH source and the ventral limit of the PAX7 domain in the midbrain at H&H 10, when midbrain patterning is ongoing, is approximately 180 \( \mu \)m. Based on our dye-coupling experiments (Fig. 3.3F), the average cell diameter of midbrain neurepithelial
Figure 3.8. Summary of HH function in the ventral midbrain shown in cartoons of wholemounts (A-D) and cross-sections (E) (A-C). A 3-step temporal patterning of the ventral midbrain by HH. (A). ≤H&H 11: Anterior-medial rFP patterning is complete or HH-independent. Caudo-medial rFP, lateral rFP and all other midbrain cell fates still require HH signaling for their specification. Increased cell spread is noted. (B). H&H 11-13: Medial rFP specification is complete. Lateral rFP and arcuate cell-fates, represented here by PHOX2A, PAX6 and EVX1 continue to be specified. Caudo-medial midbrain requires HH signaling for a longer time than rostral midbrain. HH is also required for forming a coherent arc pattern. (C). >H&H 13: Midbrain patterning is complete with the exception of lateral rFP and cell-fates associated with this region (e.g. arc 2). Ectopic cell spread is noted only in this region. (D). HH signaling regulates midbrain boundaries. Disruption of midbrain’s boundaries results in the non-autonomous spread of PAX7+ (green) cells at the DV boundary and of WNT1+ cells (blue) and FGF8+ (brown) cells at the MHB. Ectopic PAX7 (arrow) is also seen in ventral midbrain progenitors as a result of respecification into dorsal phenotypes. (E). PHOX2A expression demonstrating that Ptc1Δloop2 electroporations (right of vertical line) result in increased cell proliferation (expanded ventricular layer) and reduced differentiation compared to controls (left of vertical line). The effects are seen within columns of cytoplasmically connected midbrain cells (white), which line up ventricular to the Ptc1Δloop2+ cells (blue circles). Non-autonomous cell spread (*) is also seen. Abbreviations: 1, 1st arc; 2, 2nd arc; III, 3rd ventricle; E1, EVX1; EP, electroporation; H&H, Hamburger & Hamilton; MHB, midbrain-hindbrain boundary; P6, PAX6; rFP, rostral floor plate; TEC, tectum.
cells at H&H 10 are ~7.5 µm (range 5-10 µm; data not shown). Thus, at H&H 10, the SHH signal must travel up to ~24 cell diameters to influence cell-fates at the lateral periphery of ventral midbrain. This distance increases to ~31 cell diameters at H&H 13, which is only 1.5 times the distance of 12-20 cell diameters traversed by the HH signal in the fly wing, vertebrate limb and the spinal cord (Ericson et al., 1996; Briscoe et al., 2001; Wijgerde et al., 2002; Harfe et al., 2004; Tabata and Takei, 2004). Thus, despite the ultimately different size of the midbrain and spinal cord, the problem of getting the HH signal across long distances is circumvented by accomplishing midbrain cell-fate specification relatively early, when the midbrain size is small and comparable to the spinal cord. The role of continued \(\text{SHH}\) expression beyond this time point is not known, although cell survival, axon guidance, dorsal patterning and size regulation are possible functions (Ishibashi and McMahon, 2002; Blaess et al., 2006).

**HH signaling regulates cell cycle and differentiation in the developing midbrain**

Blockade and overexpression experiments demonstrate that HH regulates midbrain size by preventing cell proliferation and inducing differentiation with no significant alterations in cell survival (Fig. 3.2). Though midbrain size regulation in the chick midbrain following Ptc\(^1\)\(^{\text{loop2}}\) manipulations differs from that reported for the \(\text{Shh}^{-/}\) mouse, it strongly resembles the phenotype of the \(\text{Gli2};\text{Gli3}^{-/}\) and \(\text{Smo};\text{Gli3}^{-/}\) mouse spinal cords where no HH signaling is possible (Chiang et al., 1996; Ishibashi and McMahon, 2002; Wijgerde et al., 2002; Bai et al., 2004; Blaess et al., 2006). Why size regulation differs between these two sets of mice is not clear, but may depend upon the levels of GLI repressor present in each manipulation (Cayuso et al., 2006) and also upon the ligand-independent interactions between the cell cycle and HH pathway members.
Interestingly, HH signaling in the retina and cerebellar granule cells regulates multiple aspects of proliferation and differentiation, e.g. G1/S transition, cell cycle exit and neuronal differentiation (Wechsler-Reya and Scott, 1999; Pons et al., 2001; Duman-Scheel et al., 2002). Thus, whether HH is a positive or negative regulator of size may depend upon the cellular context and the level of the HH signaling cascade at which a given HH perturbation is targeted (Masai et al., 2005; Neumann, 2005).

**HH blockade results in increased cell scatter and disrupts the midbrain arc pattern**

Increased cell-scatter and a disruption of the arc pattern followed Ptc1\(^{\Delta\text{loop2}}\) electroporations in the ventral midbrain (Fig. 3.1; Fig. 4G,H). Similar disruptions in spatial patterning have also been seen following HH perturbations in multiple systems in the fly, mouse and chick (Lawrence, 1997; Litingtung and Chiang, 2000; Wijgerde et al., 2002; Bai et al., 2004; Agarwala et al., 2005). In the chick midbrain, spatially inappropriate cell-fates appeared both in radial association with Ptc1\(^{\Delta\text{loop2}}\) + cells as well as non-autonomously (e.g. Fig. 3.1H; Fig. 3.4G,H). Since robust Ptc1\(^{\Delta\text{loop2}}\) transgene was seen at E5-E6 (e.g. Fig. 3.1B, Fig. 3.4G), the selective shutdown of transgene expression in subgroups of manipulated cells is an unlikely explanation for the dual phenotype. We noted that cell-mixing/movement across midbrain boundaries (MHB, DV) following HH blockade invariably occurred in a non-autonomous manner (Figs 3.5, 3.6). Thus, a possible explanation for this dual phenotype is that it represents a combination of cell-spread (non-autonomous) and cell-fate respecification in radial association with Ptc1\(^{\Delta\text{loop2}}\) + cells.

Previous studies have noted a cell-autonomous, stepwise dorsalization of cell-fates and a non-autonomous, stepwise dorsal to ventral transformation of cell-fates due
to a failure of \( \text{Ptc}^{\Delta_{\text{loop2+}}} \) cells to sequester HH (Briscoe et al., 2001). However, in the midbrain, the non-autonomous effects were non-directional, affected progenitors and differentiated neurons and increased dramatically with time (Fig. 3.1l, Supplementary Fig. 3.7A). Thus, we interpret our findings as increased cell spread rather than a dorsal to ventral respecification due to the failure of \( \text{Ptc}^{\Delta_{\text{loop2}}} \) to bind the HH ligand.

**HH regulates midbrain’s boundaries with adjacent tissues**

In this study, we show that a consequence of HH blockade in the midbrain is increased cell proliferation resulting in a broadened MHB across which cell mixing can occur (Vaage, 1969; Kiecker and Lumsden, 2005; Zervas et al., 2005). Recent evidence suggests that rather than being a single boundary, the MHB may be a compartment flanked by two boundaries, much like the ZLI in the diencephalon (Kiecker and Lumsden, 2005). The MHB is sharpened over time through the mutual repression of OTX2 and GBX2 (Zervas et al., 2005). Taken together with our observations, these results support a role for HH signaling in sharpening the MHB by inhibiting cell proliferation. Furthermore, although controversial, the MHB is likely to be a lineage-restriction boundary, like rhombomeric boundaries, is somewhat “leaky” and permits a limited amount of cell mixing (Fig. 3.5C,D; Jungbluth et al., 2001; Zervas et al., 2005). The increased cell mixing noted across the MHB following HH blockade in our experiments therefore suggests a role for HH signaling in limiting such cell-mixing. This is corroborated in the \( \text{Shh}^{-/-} \) mouse where MHB cells can be found scattered several cell-diameters away from the MHB (Fogel et al., 2008).

The requirement for HH in boundary maintenance is not confined to the MHB. In Fig. 3.6, we noted that the DV boundary and the accompanying \( \text{SERRATE1} \) expression
are also perturbed as a consequence of HH blockade and result in cell mixing. No patterning properties are ascribed to the midbrain DV boundary yet, but SERRATE and NOTCH-DELTA interactions have been implicated in DV patterning in the fly and vertebrate limb and in the establishment of the AER, a signaling center at the DV interface (Irvine and Vogt, 1997). We conclude that maintaining the integrity and the signaling properties of boundary regions, and therefore the territorial integrity of ventral midbrain, is an important function of HH signaling.

**Radial patterning and the cell autonomy of HH action within ventral midbrain**

In Fig. 3.3, we showed that the specification of appropriate cell fates was not only blocked within the Ptc1\textsuperscript{\Lambda loop2+ cells but also in columns of Ptc1\textsuperscript{\Lambda loop2- cells radially aligned with them. In EGFP electroporations, we show that cells within a single midbrain column can be cytoplasmically continuous, raising the possibility of the transfer of small, undetectable amounts of Ptc1\textsuperscript{\Lambda loop2 between these cells to block fate specification. In the cortex, lineally related cells occupy similar radial columns and are cytoplasmically connected via gap junctions (Chenn and McConnell, 1995; Noctor et al., 2001). Intriguingly, gap junctions are also found among midbrain progenitors (Fig. 3.3F). A recent in vitro study has elegantly demonstrated the involvement of PTC1-mediated induction of provitamin D3 in suppressing HH signaling in juxtaposed cells (Bijlsma et al., 2006). This model supports the extracellular transport of provitamin D3 in the non-autonomous blockade of SMO in adjacent cells. However, provitamin D3 is a small molecule (M.W. 384.6 Daltons) and could pass via gap junctions from an electroporated cell to its cytoplasmically connected neighbors to block cell-fate specification. Thus, while the radial organization of the midbrain may depend upon the alignment of clonally
related cells, their cytoplasmic connections may help explain why they share similar fates following HH blockade.
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Chapter 4: FOXA2 and SHH are Differentially Required for the Specification and Morphology of Midbrain Floor Plate

SUMMARY

Previous work from our laboratory has shown that important midbrain cell types such as the oculomotor complex and dopaminergic neurons (MDA) depend critically upon the signaling molecule Sonic Hedgehog (SHH) secreted from a specialized ventral midline signaling center called floor plate (FP). Thus, it is ultimately the specification of the FP that is responsible for patterning neuronal cell fates within the midbrain. Interestingly, we have found that the chick midbrain FP can be divided into medial (MFP) and lateral (LFP) regions on the basis of gene expression and mode of induction. Thus, while SHH is expressed throughout FP, several developmental control genes (i.e. LMX1B, BMP7) are exclusively expressed in the MFP. Additionally, we show that HH signaling is necessary and sufficient for the specification of the LFP, but not the MFP after HH 5. We next asked what additional signals might be required for the specification of MFP and what functional differences may exist between MFP and LFP. The winged-helix transcription factor FOXA2/HNF3β is expressed throughout the midbrain FP and we show that it is sufficient to initiate the entire midbrain pattern, including MFP, which cannot be induced by SHH alone. We show that FOXA2 and not SHH misexpression creates ectopic “hinges” in the midbrain neurepithelium that resemble the endogenous ventral midline hinge point and that the ectopic induction of MFP is accompanied by a robust increase of MDA progenitors and neurons. Thus, we bring new insight into the mechanism by which midbrain FP is specified and ascribe
functional roles to its subregions. We propose that while the MFP regulates the production of dopaminergic progenitors and changes in cell-shape required for bending and shaping the neural tube, the LFP appears to be largely responsible for cell survival and the formation of a spatially coherent pattern of midbrain cell fates.
INTRODUCTION

During development, the ventral midline of the neural tube is occupied by a specialized signaling center, called the floor plate (FP), whose anatomy and function have been studied for over a century (His, 1888; Kingsbury, 1920; Strahle et al., 2004; Placzek and Briscoe, 2005). These studies have established the FP as an organizer region expressing developmental control signals which regulate many key events involved in patterning the neural tube. An important function of the FP is the secretion of the morphogen sonic hedgehog (SHH), which plays a critical role in ventral cell fate specification and regulation of size in the vertebrate neural tube (Ericson et al., 1995; Marti et al., 1995a; Jessell, 2000; Agarwala et al., 2001; Jacob and Briscoe, 2003; Bayly et al., 2007). Signaling molecules emanating from the FP (e.g. SHH, netrins, wnts) also act as axon guidance cues, directing growing axons toward, across, or away from the ventral midline of the neural tube (Colamarino and Tessier-Lavigne, 1995; Charron et al., 2003; Salinas and Zou, 2008). The FP is also required for the formation of the median hinge-point along the ventral midline of the neural plate, an event critical to bending, shaping and ultimately closing the neural tube, although the identity of molecules involved in coordinating this process remains largely unknown (Smith and Schoenwolf, 1997).

Given these critical patterning functions, a significant effort has been made to understand the development and patterning abilities of the FP, with important differences emerging between amniotes and anamniotes (Odenthal et al., 2000; Strahle et al., 2004; Placzek and Briscoe, 2005). In particular, considerable controversy exists with regard to the embryonic origin of FP, and the contribution of SHH and other patterning molecules
to its induction. SHH is expressed in the organizer (the node or its equivalent), its axial mesodermal derivatives (the notochord and prechordal plate) and the overlying FP (Marti et al., 1995b). The importance of HH signaling in FP specification has been examined in the mouse and chick. The FP is not specified in mice lacking Shh, its obligate effector Smoothened (Smo), or members of the HH signaling cascade such as the zinc finger transcription factor, GLI2 (Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998). Conversely, the entire neural tube is converted into FP-like tissue in Ptc1−/− mice where HH signaling becomes constitutively active (Goodrich et al., 1997). Taken together with gain- and loss-of-function studies in the chick, these studies support a critical role for SHH in inducing the overlying midline of the neural plate to a FP fate (van Straaten et al., 1985; Roelink et al., 1994; Charrier et al., 2002).

A more complex bipartite model of FP development has begun to emerge in anamniotes, where multiple criteria, e.g., gene expression, embryonic origin, mode of induction and function, support a subdivision of the FP into medial (MFP) and lateral (LFP) territories (Odenthal et al., 2000; Strahle et al., 2004). In the zebrafish, fate mapping studies have revealed that cells in the MFP and LFP cells likely emerge from the embryonic shield and the neurectoderm, respectively (Halpern et al., 1995; Shih and Fraser, 1995; Melby et al., 1996). Unlike the Shh−/− mice, which fail to specify FP, HH pathway mutants (sonic you, smoothened, iguana, detour) in the zebrafish only exhibit defects in LFP specification while MFP specification appears to be correctly initiated (Chiang et al., 1996; Schauerte et al., 1998; Appel, 2000; Odenthal et al., 2000; Strahle et al., 2004; Norton et al., 2005; Fogel et al., 2008). Conversely, MFP, but not LFP, specification is defective in mutants of the Nodal signaling pathway [e.g. cyclops (cyc),
one eyed pinhead (oep), foxh1/Schmalspur (sur)] (Hatta et al., 1991; Strahle et al., 1997b; Pogoda et al., 2000; Strahle et al., 2004).

Recent evidence from quail-chick chimeras suggests that a bipartite organization of the FP may not be unique to the anamniotes. When the Hensen’s node of a chicken embryo was replaced with its quail counterpart, it was observed that both the MFP and the NC were derived from the quail Hensen’s node (Catala et al., 1996). Interestingly, pan-neuronal markers (e.g., SOX1) are not expressed in the MFP although they are expressed in the LFP and throughout the neurectoderm (Charrier et al., 2002). Furthermore, a complete FP (MFP and LFP) can be obtained by the inductive action of either an ectopic notochord or an MFP, while grafts of the LFP or SHH-producing cells elicit only the induction of an ectopic LFP (Charrier et al., 2002). These experiments suggest that HH-independent and dependent pathways may be involved in the specification of the spinal FP in both amniotes and anamniotes.

Clues to the identity of HH-independent signals in MFP specification in the chick have emerged from studies in anterior FP (Dale et al., 1999; Patten et al., 2003). These studies have shown that some cells of the anterior MFP (e.g. anterior hindbrain, midbrain) originate in “area a,” an area of the epiblast anterior to the node (Schoenwolf and Sheard, 1990; Patten et al., 2003). In vitro gain-of-function studies show that “area a” cells are specified to an MFP fate by a brief combined exposure to Shh and Nodal, presumably derived from the prechordal plate (PCP; Patten et al., 2003). Thus, despite shared features, the organization and development of the FP differs not just across species, but also along the mediolateral axis and across axial levels of the neural tube.

Insight into the differences in FP specification between multiple organisms may come from an examination of how the winged-helix transcription factor, HNF3ß/FOXA2
is regulated. In multiple species, Foxa2 is expressed in the node (or its equivalent), axial mesoderm, and in a broad region along the ventral midline, encompassing both the MFP and LFP (Ruiz i Altaba et al., 1993b; Sasaki and Hogan, 1994; Odenthal et al., 2000). Despite this interesting expression pattern, loss-of-function analyses of Foxa2 in FP development has not been conducted in amniotes as Foxa2−/− mice fail to form a node and consequently, axial mesoderm (Ang and Rossant, 1994; Weinstein et al., 1994). However, ectopic expression of Foxa2 in the neural tube of transgenic mice results in the induction of many FP genes, including Shh and Foxa2 (Sasaki and Hogan, 1994). Foxa2 is also a target of HH signaling and Foxa2 consensus binding sites are present within the enhancers of mouse and zebrafish Shh genes (Sasaki and Hogan, 1994; Chang et al., 1997; Hynes et al., 1997; Epstein et al., 1999; Muller et al., 1999; Agarwala et al., 2001; Jeong and Epstein, 2003). Consequently, Foxa2 is absent from the neural tube of mice lacking HH or GLI2 activity (Chiang et al., 1996; Ding et al., 1998; Matise et al., 1998). Unfortunately, their mutual interdependence has made an analysis of their separate roles in FP specification difficult.

Mutant analyses in the zebrafish has shed light on the possible separate functions of shh and foxa2. Here foxa2 expression appears to be under complex regulation, with MFP and LFP expression depending upon Nodal and HH activity, respectively (Schauerte et al., 1998; Odenthal et al., 2000). Nodal signaling can induce foxa2 expression and ectopic foxa2 can rescue the MFP in Nodal pathway mutants (Muller et al., 2000; Rastegar et al., 2002). Unlike the mouse, where Foxa2 transcriptionally activates Shh in addition to being a transcriptional target of HH signaling, studies in the zebrafish suggest that foxa2 functions in both the nodal and the HH signaling cascades to participate in the induction of both an MFP and an LFP.
(Strahle et al., 2004). Analyses of zebrafish foxa2 (axial, monorail) mutants reflect this duality of FOXA2 function (Appel et al., 1999; Norton et al., 2005). In these mutants, an MFP is initiated, but fails to fully differentiate and express markers of later development, sharing features of both the MFP and the LFP (Appel et al., 1999; Norton et al., 2005). Additionally, ectopic induction of HH target genes is noted in a small proportion of these mutants, suggesting that FOXA2 acts as a suppresser of HH activity outside the FP. An additional observation made by these authors is that Nodal-dependent induction of MFP can occur in the absence of FOXA2, but later FP (MFP and LFP) development depends heavily on its activity (Norton et al., 2005).

Many classical studies have suggested that the midbrain FP is a unique entity, distinct from both the diencephalic ventral midline and caudal FP (Kingsbury, 1930; Sanders, 2001; Placzek and Briscoe, 2005). To distinguish it from the FP at other axial levels, this study will refer to the midbrain FP as rostral FP (rFP). The unique features of the rFP include its relatively massive size, its complex gene expression profile, the presence of multiple specialized cell types and its neurogenic potential (Placzek and Briscoe, 2005). An important set of neuronal precursors that emerge from the rFP are the midbrain dopaminergic neurons (mDA) which are implicated in addictive behaviors and in the regulation of voluntary movement (Hynes et al., 1995; Simeone, 2005; Ang, 2006; Ono et al., 2007; Bonilla et al., 2008). In addition, in vivo gain- and loss-of-function studies have established that HH signaling is either necessary and/or sufficient for the specification of multiple ventral midbrain cell types which include the rFP itself, the oculomotor complex (OMC) and the red nucleus (RN) (Agarwala et al., 2001; Agarwala and Ragsdale, 2002; Blaess et al., 2006; Bayly et al., 2007; Fogel et al., 2008).
Few studies have examined the organization and function of the rFP. Our previous studies have shown that while HH blockade prevents the specification of the LFP, the MFP is refractory to such manipulations (Bayly et al., 2007). Furthermore, the LFP is compromised, but the MFP remains intact in the midbrain of the chick talpid², a HH pathway mutant (Agarwala et al., 2005). These studies suggest that HH signaling is both necessary and sufficient for LFP specification, while its involvement in MFP specification remains unexplored. Gene expression studies have suggested that such a subdivision may also be possible in the mammalian FP (Ruiz i Altaba et al., 1993a; Sasaki and Hogan, 1994; Odenthal et al., 2000). However, an examination of Foxa2 function in the mouse and the fish is complicated due to the presence of Foxa1, a partially redundant homolog, in the FP of each species. This problem is circumvented in the chick, as FOXA1 is not present according to the most recent genome annotation, and where regional manipulations of FOXA2 can be conducted without compromising the formation of the node and node-derived mesodermal tissues.

In this study, we have compared the role of FOXA2 and SHH in FP specification at 2 axial levels: the midbrain and the spinal cord (Charrier et al., 2002; Agarwala et al., 2005; Bayly et al., 2007). We show that FOXA2, but not SHH, is necessary and sufficient to execute the entire FP program (MFP and LFP) in both the midbrain and spinal cord. Additionally, we show that by inducing SHH, FOXA2 fully replicates HH activity in multiple assays, including cell fate specification, growth control and differentiation. We have found that unlike SHH, FOXA2 overexpression can a) induce an MFP, b) result in the hinging of the neural tube in a manner reminiscent of that observed at the ventral midline during neurulation and c) induce ectopic proliferation in the mantle layer not observed with SHH misexpression. We show that HH-independent
regulation of BMP, rather than Nodal signaling, is most likely to account for the activities of FOXA2 that do not overlap with SHH. Finally, we show that although SHH is necessary for the specification of mature mDA neurons, it can only induce mDA progenitors ($LMX1A^+$, $LMX1B^+$, $NURR1^+$, $PITX3^+$) in a modest and regionally restricted manner. By contrast, FOXA2 is a potent inducer of the entire mDA program throughout the midbrain. Our results thus suggest a broad role for FP specification by FOXA2, which is likely to be executed by the regulation of SHH and BMP signaling.
RESULTS

Gene expression patterns suggest a complex subarchitecture for the midbrain floor plate and a role for FOXA2 in its specification

Studies in the zebrafish and the chick spinal cord have suggested that the FP can be divided into medial (MFP) and lateral (LFP) subdivisions based on several criteria including cell-type composition, gene expression patterns, mode of specification and embryonic origin (Charrier et al., 2002; Strahle et al., 2004). Given the large size of the midbrain and the distinctiveness of the FP at different axial levels, we asked whether midbrain/rostral FP (rFP) develops in a manner similar to the spinal cord. Sonic hedgehog (SHH) is expressed along the embryonic midline by Hamburger and Hamilton (H&H) stage 3, and is clearly seen in the rFP by H&H 7-8 (Fig. 4.1A; Hamburger and Hamilton, 1951; Lawson et al., 2001; Patten et al., 2003; Aglyamova and Agarwala, 2007). At this time, the expression of the LIM homeodomain transcription factor, LMX1B, overlaps with that of SHH along the ventral midline of the midbrain in addition to the prospective midbrain-hindbrain boundary (MHB; Fig. 4.1A; Yuan and Schoenwolf, 1999). Interestingly, analysis at a higher level of magnification revealed that the levels of coexpression between LMX1B and SHH were highly mosaic (Supplementary Fig. 4.18A). By H&H 15, an SHH-positive (SHH+)/LMX1B-negative (LMX1B-) rostral territory encompasses the ventral midline (SHH+/LMX1B+) rFP (Fig. 4.1B, arrowhead). Based on multiple criteria (see below), we have identified the medial (SHH+/LMX1B+) territory as the midbrain MFP and the lateral territory (SHH+/LMX1B-) as the LFP. Between late E3 and E6, the LFP extends caudalward toward the MHB and fully encompasses the MFP (Fig. 4.1C; data not shown). Thus, a distinction between the MFP and the LFP is
first noted at H&H 15 and is maintained at least until E6. In addition to LMX1B, multiple markers, including the HH effector, DISP1, and the Bone Morphogenetic Protein (BMP) family member, BMP7, are also expressed exclusively in the LMX1B+ territory and distinguish it from the LFP (Fig. 4.1D, E; Fogel, 2009). Further examination of rFP gene expression patterns suggests that the subarchitecture of the rFP may be more complex. For example, BMP5 and the BMP antagonist Chordin (CHD), are expressed narrowly along the ventral midline in a region circumscribed by LMX1B expression at E4 (Fig. 1F; Fogel, 2009). However, since our perturbations appeared not to selectively affect this region, we have treated the MFP as one entity until further study (Bayly and Agarwala, unpublished observations).

Foxa2/monorail plays an important role in zebrafish FP specification, interacting with both the nodal and HH signaling cascades to specify the MFP and the LFP, respectively (Norton et al., 2005). The early expression of foxa2, together with gain- and loss-of-function analyses in mutant mice strongly suggests an important role in FP specification (Ruiz i Altaba et al., 1993a; Ang and Rossant, 1994; Sasaki and Hogan, 1994; Weinstein et al., 1994). However, due to the absence of a node and its axial mesodermal derivatives, understanding the winged-helix transcription factor, FOXA2/HNF3β, in FP specification and pattern formation through null mutant analyses has been difficult (Ang and Rossant, 1994; Weinstein et al., 1994). To examine FOXA2 function in FP development and determine whether it differed from that of SHH, we examined their expression patterns between H&H 3+ and E6. The onset of FOXA2 coincides with that of SHH in the chick axial midline at H&H 3+ and also like SHH, FOXA2 is expressed throughout the FP region (MFP and LFP) as well as in a territory lateral to it (Fig. 4.1G, H, I; data not shown; Ruiz I Altaba et al., 1993b; Lawson et al.,
Figure 4.1. Gene expression patterns suggest a complex subarchitecture for the midbrain floor plate and a role for FOXA2 in its specification

(A). Wholemount preparation of a HH 8 embryo oriented so that rostral is to the top and the ventricular surface faces the viewer. LMX1B expression (blue) is coexpressed with SHH (brown) in the rFP of the midbrain as well as diffusely present in the presumptive MHB. (B). By H&H 15, LMX1B expression (blue) remains restricted to the medial region of the FP, while SHH expression (brown) begins to expand laterally while moving in a caudalward direction. (C). By E5, an LMX1B+/SHH+ MFP territory becomes distinct from that of the LMX1B-/SHH+ LFP. (D). While LMX1B expression (brown) is observed within the MFP and the MHB, DISP1 expression (blue) is entirely restricted to the MFP and coexpressed with LMX1B. (E). BMP7 expression (blue) is also observed within the MFP, suggesting a potential role in its specification (Fogel, 2009). (F). In addition to secreted BMP ligands, BMP antagonists such as CHORDIN (blue), are expressed within the MFP and perhaps delineate a more complex subarchitecture of the midbrain floor plate. (G-I). FOXA2 expression (brown) is coincident with SHH (blue) throughout the development of the floor plate. Abbreviations: HH: Hamburger & Hamilton stage; LFP: lateral floor plate; MFP: medial floor plate; MHB: midbrain-hindbrain boundary; rFP: rostral floor plate.
Like LMX1B, the levels of coexpression of FOXA2 and SHH were highly mosaic in appearance, perhaps indicating a mixed population of cell types (Supplementary Fig. 4.18B). In addition to its expression in the floor plate, FOXA2 is also observed in differentiated ventral midbrain neurons. These include neurons lateral to the rFP and a class of differentiated LMX1B+ dopaminergic (DA) progenitors (Supplementary Fig. 4.18C).

Differential requirement for HH signaling in the specification of medial and lateral floor plate

Studies in the zebrafish have shown that the disruption of HH signaling prevents the specification of LFP but does not affect MFP specification (Schauerte et al., 1998; Appel, 2000; Odenthal et al., 2000; Strahle et al., 2004). We previously demonstrated that while the specification of midbrain neural progenitors and the lateral regions of the rFP have an absolute requirement for HH signaling, the medial region of the rFP was largely refractory to HH blockade (Fig. 3.4B, C; Bayly et al., 2007). To determine whether the differentially affected medial and lateral regions corresponded to the MFP and LFP territories identified through our gene expression analyses, we examined the expression patterns of MFP (LMX1B+/DISP1+/SHH+) and LFP (LMX1B-/DISP1-/SHH+) markers following HH blockade. We found that ubiquitous, bilateral misexpression of Ptc1Δloop2, a dominant negative receptor of HH signaling, between H&H 7-21 resulted in a robust disruption of the LFP while leaving the MFP intact (Fig. 4.2A, arrowhead, compare to Fig. 4.1C; data not shown; Bayly et al., 2007). Interestingly, examination of rFP specification in the chick talpid2, a naturally occurring HH pathway mutant, revealed a severely disrupted LFP that was accompanied by an intact MFP, closely mimicking
Figure 4.2. Differential requirement for HH signaling in the specification of medial and lateral floor plate

(A). Ubiquitous, bilateral misexpression of \( \text{PTC}_{1}^{\text{loop2}} \) disrupt the lateral regions of floor plate (\( \text{SHH} \), brown, arrowhead), but did not affect the medial region of floor plate (\( \text{LMX1B} \), blue, \( \text{SHH} \)).  

(B). In the \( \text{talpid}^{2} \), a naturally occurring HH pathway mutant, the lateral regions of floor plate (\( \text{SHH} \), brown, arrowhead) are disrupted while the medial region of floor plate (\( \text{LMX1B} \), blue, \( \text{SHH} \)) remains intact.  

(C). Control explants of H&H 7 midbrains reveal the normal restriction of \( \text{PAX7} \) expression (blue) to the dorsal edges of the tissue.  

(D). In the presence of cyclopamine for 24 hours, \( \text{PAX7} \) expression (blue) occupies a more ventral region of the explants although is absent from the ventral midline.  

(E). In the absence of cyclopamine, H&H 5 whole embryo explants reveal the presence of \( \text{LMX1B} \) (brown) and \( \text{PTC1} \) (blue, arrowhead) in the prospective midbrain.  

(F). In the presence of cyclopamine, H&H 5 whole embryo explants lack \( \text{PTC1} \) expression (blue), yet the expression of \( \text{LMX1B} \) (brown) along the rFP is unaffected.

what was observed after \( \textit{Ptc1}\text{\textsuperscript{-loop2}} \) electroporations (Fig. 4.2B, arrowhead; Caruccio et al., 1999; Agarwala et al., 2005). These results suggest that like the FP at spinal cord levels, the rFP can also be divided into medial and lateral subdivisions based on their dependence upon HH signaling (Charrier et al., 2002). However, protein expression is first observed from the pCIG-\( \textit{Ptc1}\text{\textsuperscript{-loop2}}\)-IRES-EGFP vector 4-6 hours after electroporation and therefore electroporation at H&H 7 might not effectively block HH signaling until as late as H&H 9-10 (29-33 hours post-incubation; personal observations; Hamburger and Hamilton, 1951; Chen et al., 2004). To avoid the possibility of an early requirement of HH signaling in the establishment of the MFP, we resorted to a midbrain explant culture system using cyclopamine, a fast acting inhibitor of HH signaling (Incardona et al., 1998). The exclusion of the dorsal midbrain marker \( \textit{PAX7} \) from ventral midbrain demonstrated that dorsal and ventral midbrain was properly regionalized in H&H 7-9 control explants (\( n=6/6 \); Fig. 4.2C; Ericson et al., 1997). Bath application of cyclopamine (100 µM) for 24 hours to midbrain explants resulted in ectopic \( \textit{PAX7} \) expression in ventral midbrain consistent with our previous findings, however, the ventral midline (presumptive MFP) was invariably found to be \( \textit{PAX7} \)- (\( n=4/4 \); Fig. 4.2D; Bayly et al., 2007). Furthermore, in H&H 5 explants, while the application of cyclopamine successfully blocked the expression of \( \textit{PTC1} \) (\( n=5/5 \), arrowhead), a transcriptional target of HH signaling, it failed to block \( \textit{LMX1B} \) expression in the MFP (\( n=5/5 \); Fig. 4.2E, F). Taken together, these results suggest that like the FP of the spinal cord, rFP can also be divided into medial and lateral subdivisions based on their differential dependence on HH signaling (Charrier et al., 2002).
**SHH is only sufficient to induce the MFP fate in a regionally restricted manner**

Although HH signaling at and after H&H 5 does not appear to be necessary for the specification of the MFP, we asked whether it was sufficient. We previously showed that misexpression of *SHH* in the chick midbrain can induce an *SHH*+ rFP and recapitulate the ventral midbrain pattern of cell fates (Agarwala et al., 2001; Agarwala and Ragsdale, 2002). Here we determined whether both the MFP and the LFP could be induced as a consequence of *SHH* misexpression. Indeed, misexpression of *SHH* resulted in the widespread induction of ectopic *SHH* throughout the midbrain, suggesting that these manipulations converted large swaths of midbrain into rFP (Fig. 4.3A). However, regardless of its concentration (1-5 mg/ml), electroporated *SHH* could only ectopically induce *LMX1B* along the MHB and nowhere else in the midbrain (Fig. 4.3A, B; data not shown). Additionally, *DISP1*, whose expression is normally restricted to the MFP, was coexpressed with *LMX1B* along the MHB after *SHH* misexpression, suggesting a conversion of the MHB to an MFP fate, rather than an expansion of the MHB (Fig. 4.3B-D). Simultaneously, ectopic *SHH* suppressed markers of the MHB (*FGF8, WNT1*), confirming that the MHB program was suppressed and converted into an MFP fate (Fig. 4.3C-E). The conversion of the MHB to MFP after *SHH* overexpression could also occur away from the endogenous MFP suggesting that an induction event, rather than the expansion of the MFP was taking place (Fig. 4.3D, arrowhead). Although *FGF8* and *WNT1* were suppressed, the anteroposterior (A/P) boundary between *OTX2* and *GBX2* expression was unaffected, suggesting that the segregation and regional identity between the midbrain and hindbrain remained intact (Fig. 4.3F; Jungbluth et al., 2001). Together, these results suggest that SHH is necessary and
Figure 4.3. SHH is only sufficient to induce the MFP fate in a regionally restricted manner

(A). Unilateral expression of SHH is able to convert a large majority of ventral midbrain into a SHH+ (brown) floor plate, although only induced LMX1B expression (blue) along the MHB.  (B). The induction of LMX1B (brown) along the MHB after misexpression of SHH is coexpressed with DISP1 (blue), a marker specifically expressed within the MFP.  (C, D). The induction of MFP (DISP1, blue) along the MHB after misexpression of SHH coincides with the suppression of signaling molecules that emanate from the MHB (FGF8, brown, C), which may or may not be continuous with the endogenous MFP territory (D, arrowhead).  (E). In addition to the suppression of FGF8, SHH overexpression (brown) is also sufficient to suppress WNT1 expression (blue).  (F). Although both FGF8 and WNT1 are suppressed in the presence of SHH, the anteroposterior boundary between midbrain (OTX2, blue) and hindbrain (GBX2, brown) remains unaffected.  Abbreviations: EP: electroporation; HB: hindbrain; MB: midbrain; MHB: midbrain-hindbrain boundary.
sufficient for the specification of the LFP, but is only sufficient to specify MFP in a regionally restricted area of the prospective midbrain.

**FOXA2 is sufficient to induce medial floor plate throughout the ventral midbrain**

To our knowledge, FOXA2 is the only gene aside from SHH that is expressed in the node, its derivatives and subsequently in the entire rFP between H&H 3+ to E6 (Fig. 4.1; Ruiz i Altaba et al., 1993a; Sasaki and Hogan, 1993; Lawson et al., 2001; Aglyamova and Agarwala, 2007). Despite this expression pattern, the lack of a discernable node and notochord in *Foxa2*/*-* mutants has made it difficult to understand how FOXA2 functions in the specification of the rFP in amniotes (Ang and Rossant, 1994; Weinstein et al., 1994). In the zebrafish however, FOXA2 function differs from that of SHH, affecting MFP and LFP specification in addition to the maturation of the FP (Appel, 2000; Strahle et al., 2004; Norton et al., 2005). Here we analyzed FOXA2 gain- and loss-of-function experiments between H&H 8-11 at a time when the rFP is still being induced, but following the specification of the node, the prechordal plate and notochord, so that they are not compromised.

Like *SHH*, overexpression of pMES-FOXA2-IRES-EGFP between H&H 8-11 autonomously induced SHH, resulting in the non-autonomous induction of *SHH* by itself in adjacent tissue (Fig. 4.4A; Supplementary Fig. 4.18D). Unlike *SHH* however, misexpression of FOXA2 was also able to induce MFP markers (*LMX1B* and *DISP1*) throughout the ventral midbrain and not just along the MHB (Fig. 4.4A, B, arrowheads).
Figure 4.4. FOXA2 is sufficient to induce medial floor plate throughout the ventral midbrain

(A). Bilateral misexpression of FOXA2 is sufficient to induce MFP (LMX1B, blue, arrowhead) and SHH expression (brown) away from the MHB. (B). In addition to LMX1B, forced expression of FOXA2 is also sufficient to induce DISP1 expression (blue) in an largely cell autonomous manner (arrowheads) away from the MHB and non-autonomously along the MHB (arrow). (C). CHORDIN (blue) is also induced by FOXA2 (arrowhead), but not in all cells that ectopically expressed LMX1B (brown, arrow). However, the ectopic CHD+ cells were not properly localized along the ventricular-pial axis (Fig. 4.18F). (D). Coexpression of FOXA2 with a dominant negative form of the same protein (dnFOXA2, brown, arrowhead) almost completely suppressed the normally robust induction of DISP1 expression (blue). An arrow points to a small region of ectopically induced DISP1+ cells. (E). Electroporation of dnFOXA2 was sufficient to suppress SHH expression (brown, arrowhead) and thus LFP. (F). dnFOXA2 (brown) was also sufficient to significantly prevent the specification of the MFP (DISP1, blue) in a largely cell autonomous manner (arrowhead). Abbreviations: bi: bilateral electroporation; EP: electroporation; MHB: midbrain-hindbrain boundary.
Furthermore, FOXA2 overexpression also induced a small number of ectopic LMX1B+/CHD+ cells, suggesting that the entire MFP program, including its most ventral elements, were replicated by FOXA2 overexpression (Fig. 4.4C, arrowhead). However, the ectopically induced CHD+ cells were observed far less often than LMX1B+ cells after FOXA2 misexpression and were incorrectly localized to pial surface of the tissue (Supplementary Fig. 4.18E, F). In order to analyze the requirement of FOXA2 in the specification of the MFP, we electroporated a dominant negative form of FOXA2 (dnFOXA2, see Materials and Methods; Jacob et al., 2007). To test the validity of the construct, we coelectroporated dnFOXA2 and FOXA2 with the expectation that FOXA2 would be unable to induce an MFP fate in the presence of dnFOXA2. Indeed, dnFOXA2 was sufficient to inhibit the ectopic induction of DISP1 normally observed with FOXA2 alone (Fig. 4.4D, arrowhead). Misexpression of dnFOXA2 alone resulted in the robust suppression of SHH in the LFP as well as SHH and DISP1 in the MFP (Fig. 4.4D, E, arrowheads, Supplementary Fig. 4.18G). These observations are in stark contrast to the failure of Ptc1Δloop2 and cyclopamine experiments to block MFP gene expression at all ages examined (Fig. 4.2). Taken together, our results suggest that FOXA2 is both necessary and sufficient to execute the MFP program while SHH is only sufficient to do so in a regionally restricted manner along the MHB.

**RNAi-mediated knockdown of FOXA2 occasionally leads to an expansion of the MFP territory**

In addition to the dnFOXA2 construct, we designed an RNAi expression vector targeted to FOXA2. This expression vector produces an RNA molecule that forms a hairpin loop, where one strand is complementary to a 21nt region of the FOXA2 mRNA
Figure 4.5. RNAi-mediated knockdown of FOXA2 occasionally leads to an expansion of the MFP territory (A, B). Compared to unelectroporated controls (A), bilateral misexpression of FOXA2 RNAi caused a modest and global reduction of FOXA2 expression (blue, B). The reduction of FOXA2 is most obvious in regions where it is normally present in low levels (red arrowheads). Note the overall reduction in the size of the FP after FOXA2 knockdown (double headed arrows). (C). Electroporation of EGFP does not affect the gene expression patterns of LMX1B (brown) and CHORDIN (blue). (D). Interestingly, bilateral misexpression of FOXA2 RNAi largely had no affect on LMX1B expression (brown) except in a few cases, where an expansion of the LMX1B territory was observed (n=6/25, arrowhead). However, in all cases, CHORDIN expression (blue) was reduced compared to controls (arrow). Abbreviations: E: embryonic day; EP: electroporation.
(see Materials and Methods). These small RNA molecules will recruit Dicer and degrade FOXA2 mRNA and because their expression is driven by a vector, they will continue to do so through subsequent cell divisions as the plasmid is passed on to daughter cells (Katahira and Nakamura, 2003).

Compared to normal expression, bilateral misexpression of FOXA2 RNAi between H&H 7-8 led to a moderate global reduction in the amount of FOXA2 transcript, reducing the overall size of FP, but keeping the relative pattern of FOXA2 expression intact (Fig. 4.5A, B, double headed arrows). Interestingly, although RNAi-mediated knockdown of FOXA2 caused a reduction of CHORDIN expression (n=11/15) compared to EGFP-electroporated controls, it largely had no affect on LMX1B expression (Fig. 4.5C, D). Surprisingly, in a few instances (n=6/25), a slight expansion of the LMX1B territory was observed (Fig. 4.5D, arrowhead). This observed increase of FP with reduced levels of FOXA2 was similarly reported in zebrafish monorail/foxa2 mutants suggesting a potential dual role of FOXA2 in limiting FP development possibly through a concentration dependent mechanism (Norton et al., 2005).

**FOXA2, but not SHH, is necessary and sufficient to induce a midbrain hinge-point**

One of the earliest events in neurulation is the bending of the neurepithelium along the ventral midline or FP (Schoenwolf and Smith, 2000). This morphological process occurs in part due to changes in cell-shape of specific cells that are positioned along the midline. Originally cuboidal in shape, these cells become constricted at their apical surface and their nuclei translocate basally, ultimately causing a change to a wedge-shaped morphology (Schoenwolf and Smith, 2000). As a group, these wedge-shaped cells effectively create a hinge-point forcing the lateral regions of the
neurepithelial sheet upwards and closer together (Schoenwolf and Sheard, 1990). Additionally, this observation suggests that signals emanating from the FP or the underlying notochord can regulate these morphological behaviors. Indeed, early transplantation experiments in chick have shown that placing a notochord or an MFP adjacent to more dorsal regions of the neural tube elicited the morphological behavior associated with the endogenous FP (van Straaten et al., 1985; Charrier et al., 2002). Although these observations have been known for some time, our understanding of the specific molecular mechanisms involved in this process remains unclear.

To determine whether or not the MFP markers induced by FOXA2 misexpression were also associated with ectopic hinge-points, we analyzed cell behavior in cross-section. As with the spinal cord, ectopic expression of FOXA2 in intermediate regions of the midbrain resulted in the induction of an ectopic hinge-point(s) that coincided with the ectopically induced $LMX1B^+$ MFP territory (Fig. 4.6A, B, arrowheads). Interestingly, every hinge-point was associated with ectopic $LMX1B$ expression, but not all ectopic $LMX1B$ was associated with a hinge-point (Fig 4.6B, C, arrows).

Classic embryological studies have suggested that hinge-point formation results in part from cytoskeletal reorganization and the basal migration of nuclei in cells within the neurepithelium (reviewed by Schoenwolf and Smith, 2000). A close examination of F-actin localization and the positioning of the nucleus revealed that the ectopic hinge-points produced by FOXA2 misexpression indeed possessed the behaviors associated with the endogenous median hinge-point (Fig. 4.6C-E). Interestingly, misexpression of dnFOXA2 elicited the opposite phenotype, where the normally concave apical/ventricular surface of the midbrain was converted into a convex “bulge” protruding into the lumen of the neural
Figure 4.6. FOXA2, but not SHH, is necessary and sufficient to induce a midbrain hinge-point

(A). A light micrograph taken in phase contrast of a cross-section through the midbrain after misexpression of FOXA2. As in the spinal cord, FOXA2 misexpression (EGFP+, blue) induced several ectopic hinge-points that were associated with LMX1B expression (brown). (B). Examined in a higher magnification, the presence of ectopically induced hinge-points (arrowheads) are always associated with electroporated cells (blue) ectopic LMX1B expression (brown). However, not all ectopic LMX1B expression is associated with a hinge-point (arrows, arrow in C). (C). Confocal microscopy clearly reveals that the cells electroporated with FOXA2 (GFP, green) are associated with a dramatic change in the organization of the midbrain neurepithelium as determined by F-acting (phalloidin, red) and nuclear staining (DAPI, blue). (D). A magnified view of the boxed region in C more clearly shows an increase in the amount of apically-localized F-actin where the ectopic hinge-point is present and its association with the FOXA2-electroporated cells (arrowheads). (E). DAPI-staining reveals that the electroporated cells have basally located nuclei – a hallmark cell behavior during hinge-point formation. (F). Misexpression of dnFOXA2 (GFP, green) resulted in the “flattening” of the midbrain epithelium (G, H). Intriguingly, in some instances, misexpression of dnFOXA2 (GFP, green) resulted in the formation of a “bulge,” an alteration of epithelial morphology in the opposite direction. (I-K). Unlike FOXA2 manipulations, overexpression of SHH (GFP, green, arrowheads) away from the MHB largely had no effect on the epithelial morphology of the midbrain. Abbreviations: DAPI: 4',6-diamidino-2-phenylindole; EP: electroporation; GFP: green fluorescent protein; L: lumen; PHAL: phalloidin; rFP: rostral floor plate.
tube (Fig. 4.6F-H). Additionally, and in agreement with gene expression data, \textit{SHH} misexpression could not induce ectopic hinge-points (Fig. 4.6I-K). However, along the MHB, \textit{SHH} misexpression was most commonly observed to produce a single ectopic hinge-point produced by the exaggerated bending of neurepithelium due to the conversion of the MHB to an MFP fate (data not shown, Fig. 4.3). Together with our observations in the spinal cord we propose that FOXA2 is necessary and sufficient to induce hinge-points along the neural tube, which aid in bending, shaping and closing the neural plate. By extension, our results also suggest that the initiation of the median hinge-point is a function of MFP, rather than LFP.

**FOXA2 is indirectly required for the specification of the red nucleus and the oculomotor complex**

One large distinction between the floor plate of the midbrain and that of more caudal regions of the neural tube is its neurogenic potential (Kittappa et al., 2007; Ono et al., 2007). This observation was made over 100 years ago, but until only recently has it been shown which cell types originate from within the floor plate (Kingsbury, 1930; Kittappa et al., 2007; Ono et al., 2007). A genetic cell-fate mapping strategy was used in the mouse to track the lineage of cells that at one time expressed \textit{shh}, normally restricted to cells within the floor plate (Kittappa et al., 2007). Intriguingly, they observed these cells eventually differentiate to become oculomotor and dopaminergic neurons (Kittappa et al., 2007). However, it was not reported whether every oculomotor and dopaminergic neuron originated from the floor plate or if the MFP and LFP differentially contributed to each cell type.
Figure 4.7. FOXA2 is indirectly required for the specification of the red nucleus and the oculomotor complex

(A). Unilateral misexpression of FOXA2 (EGFP, blue) is sufficient to induce oculomotor neurons (OMC; ISL1, brown) in a largely non-autonomous manner. (B). In addition to ISL1, forced expression of FOXA2 (EGFP, brown) is also sufficient to induce the red nucleus (BRN3A, blue) in a non-autonomous manner (arrowhead). (C). When FOXA2 is electroporated away from the endogenous MFP, "islands" of ectopic MFP (LMX1B, brown, arrowheads) are observed surrounded by supernumerary ISL1+ neurons of the OMC (blue, arrows). (D). FOXA2 RNAi (blue) causes a suppression of PHOX2A+ cells (brown, arrowheads), although most likely as an indirect consequence of suppressing SHH expression. 

Our previous results have demonstrated that SHH is necessary and sufficient for the specification of cell fates within ventral midbrain including the red nucleus (BRN3A+) and the oculomotor complex (OMC, ISL1+, PHOX2A+; Agarwala et al., 2001; Bayly et al., 2007). Like SHH, FOXA2 overexpression similarly elicited the ectopic induction of ISL1 and BRN3A, although it largely occurred in a non-autonomous manner (Fig. 4.7A, B, arrowheads). The non-autonomous induction of the OMC and red nucleus is likely due to the cell autonomous induction of MFP (Fig. 4.7A, B). Indeed, overexpression of FOXA2 resulted in “islands” of LMX1B+ territories surrounded by ISL1+ cells, maintaining the relative spatial relationships observed along the ventral midline (Fig. 4.7C; Sanders, 2001). Interestingly, RNAi-mediated knockdown of FOXA2 suppressed the number of PHOX2A+ cells (Fig. 4.7D). However, this is likely to be the result of a reduction in the overall production of SHH from the floor plate as SHH is a transcriptional target of FOXA2 and we have previously shown that HH blockade can achieve the same effect (Fig. 3.1E; Bayly et al., 2007). Taken together, these data suggest an early instructive role for the MFP in the initiation of pattern in the ventral midbrain as small regions of ectopic MFP are circumscribed with more dorsal cell fates.

Gene expression suggests that dopaminergic neurons originate from within the MFP

One of the many ways that the midbrain floor plate is unique from floor plate in more caudal regions of the neural tube is its ability to generate neurons (Kittappa et al., 2007; Ono et al., 2007). Mice were engineered to express LacZ in cells that expressed Shh (FP), in order to track the cell-derivatives of the floor plate. These cells that at one
time expressed Shh, identified by β-galactosidase staining, were found to populate oculomotor and dopaminergic neurons (Kittappa et al., 2007). FOXA2 and SHH are each expressed throughout midbrain FP, however, when analyzed in cross-section, differences in expression patterns emerge. At E5, SHH expression is restricted to the floor plate, whereas FOXA2 is also found in a population of differentiated cells located subjacent to the MFP (Fig. 4.8A). This population of FOXA2+ differentiated cells is also associated with expression of LMX1B, LMX1A, and NURR1, all of which have been shown to be critically important for the development of dopaminergic neurons (Fig. 4.8B-D; Zetterstrom et al., 1997; Smidt et al., 2000; Andersson et al., 2006). By E6, the amount of differentiated LMX1B+ cells has significantly increased and extends laterally along the mantle layer (Fig. 4.8E). This most lateral region of the LMX1B+ territory coexpresses TH (Fig. 4.8F). These data suggest that dopaminergic neurons (TH+ cells) are derivatives of cells that originate from within the MFP that later migrate laterally, consistent with observations in the mouse (Kittappa et al., 2007; Ono et al., 2007).

**FOXA2, but not SHH, is sufficient for the induction of dopaminergic progenitors**

The rFP has recently been shown through cell lineage analysis to constitute the majority of mDA neurons in the mouse midbrain (Kittappa et al., 2007; Ono et al., 2007). This observation, together with the differential ability of FOXA2 and SHH to specify FP, prompted us to examine their respective contributions towards the specification of mDA progenitors and neurons. LMX1A and LMX1B have been identified as early molecular markers of mDA progenitors (Figs. 4.9A, B, 4.8, data not shown; Smidt et al., 2000;
Figure 4.8. Gene expression suggests that dopaminergic neurons originate from within the MFP
(A). At E5, SHH expression (blue) is restricted to the FP, whereas FOXA2 expression (brown) is observed within a population of differentiated cells subjacent to the MFP (arrowhead). (B-D). The FOXA2+ population of differentiated cells is also associated with LMX1B (blue, B), LMX1A (blue, C) and NURR1 (blue, D), all of which are critically implicated in the specification of dopaminergic neurons. (E). By E6, the population of differentiated LMX1B+ cells (blue, E) has increased and expanded lateral to the MFP. (F). The lateral population of differentiated LMX1B+ cells is associated with the onset of TH expression (blue, F, dopaminergic neurons). Abbreviations: *: ventral midline; E: embryonic day; LFP: lateral floor plate; MFP: medial floor plate.
Andersson et al., 2006). Interestingly, the expression of both \textit{LMX1A} and \textit{LMX1B} are restricted to the MFP, suggesting that the origin of dopaminergic progenitors resides specifically within the MFP and not the LFP (Figs. 4.4A, 4.9A). Similar to \textit{LMX1B}, \textit{FOXA2} overexpression induced \textit{LMX1A} throughout ventral midbrain (arrowheads) while \textit{SHH} could only do so along the MHB in a regionally restricted manner (Fig. 4.9A, B). When analyzed in cross-section, the ectopic \textit{LMX1A} induced by \textit{FOXA2} and \textit{SHH} was localized to cells in the ventricular layer (MFP) and in the mantle layer, which presumably will become differentiated dopaminergic neurons (Fig. 4.9C, D, arrows, arrowheads; Andersson et al., 2006). \textit{PITX3} is an additional transcription factor that plays a critical role in the development of dopaminergic neurons (Zetterstrom et al., 1997; Smidt et al., 2000; Maxwell et al., 2005; Andersson et al., 2006). However, unlike the mouse, \textit{PITX3} expression in the chick is observed in the first arc in addition to the differentiated population of \textit{LMX1A+/LMX1B+} cells (Fig. 4.9E, F, arrows, arrowheads). Consistent with its pattern of expression, \textit{FOXA2} elicited the induction of \textit{PITX3} throughout the midbrain, which was localized to the intermediate region (first arc) in addition to the pial edge of the mantle layer (differentiated DA neurons) (Fig. 4.9E, F). Interestingly, unlike \textit{LMX1A} and \textit{LMX1B}, \textit{SHH} overexpression could induce \textit{PITX3} expression away from the MHB (Fig. 4.9G, arrowhead). However, this ectopic \textit{PITX3} expression was entirely restricted to the intermediate region, absent from the pial edge of the mantle layer (Fig. 4.9H, red arrowhead).

If dopaminergic progenitors originate from within the MFP, then the ectopic MFP induced by \textit{FOXA2} should be associated with a corresponding increase of \textit{Tyrosine Hydroxylase} expression (\textit{TH+}, DA neurons) throughout the midbrain whereas \textit{SHH} overexpression should only lead to an increase in DA neurons in a regionally restricted
Figure 4.9. FOXA2, but not SHH, is sufficient for the induction of dopaminergic progenitors

(A). Unilateral misexpression of FOXA2 is sufficient to induce dopaminergic progenitors (LMX1A, blue, arrowheads) throughout the ventral midbrain. (B). In a cross-section at the level shown in A, induced LMX1A expression (blue) is located in both the ventricular (progenitor, arrow) layer and the mantle (differentiated, arrowhead) layer. (C). In contrast to FOXA2 misexpression, SHH is sufficient to induce LMX1A (blue) along the MHB. (D). A cross-section shown at the level indicated in C reveals an increase in the amount of LMX1A expression (blue) in both the ventricular and mantle layer. (E). PITX3 expression (blue) is located in both the MFP (arrowhead) and the LFP (arrow). FOXA2 overexpression causes a robust increase in the amount of PITX3+ cells. (F). A cross-section at the level indicated in E reveals ectopic expression of PITX3 (blue) found along the pial surface of the mantle layer (differentiated dopaminergic cells, arrowheads) in addition to an intermediate region of the consistent with its expression in the first arc (arrows). (G). Unlike LMX1A, SHH overexpression leads to ectopic PITX3 expression (blue) away from the MHB. (H). A cross-section through the level indicated in G reveals that the ectopically induced PITX3 expression (blue) is only observed in the intermediate region of the mantle layer and not the most pial region (red arrowhead). This suggests a failure of SHH to induce dopaminergic progenitors. (I). The induction of markers associated with dopaminergic progenitors by FOXA2 is concomitant with a robust induction of TH expression (blue, dopaminergic neurons). (J). A cross-section through the level indicated in I reveals the proper localization of TH+ cells along the pial edge of the mantle layer. (K, L). Unlike FOXA2 expression, ectopic SHH is only sufficient to moderately increase the amount of TH+ expression (K, blue, dopaminergic neurons) that is properly localized (L, arrowhead). (M, N). Knockdown of FOXA2 causes a dramatic reduction in the amount of dopaminergic neurons (blue, red arrowhead) compared to the left (non-electroporated, control) side. (O). Misexpression of FOXA2 leads to the premature differentiation of the endogenous population of dopaminergic progenitors (TH, blue, arrowhead) and those that are ectopically induced (arrow) by a half day. (P). Like FOXA2, overexpression of SHH causes the premature induction of dopaminergic progenitors (TH, blue). Note that the presence of SHH away from the endogenous population of dopaminergic progenitors does not lead to ectopic TH expression (red arrowhead). Abbreviations: E: embryonic day; EP: electroporation; rFP: rostral floor plate.
manner along the MHB. Indeed, after FOXA2 misexpression, we observed a robust induction of TH+ cells throughout the ventral midbrain, which were properly localized along the pial edge of the mantle layer, consistent with observations in the mouse (Fig. 4.9I, J; Sasaki and Hogan, 1994). Unlike FOXA2, SHH misexpression only led to an increase in DA neurons along the MHB, where it had been converted to an MFP fate (Fig. 4.9K, L). Conversely, when we knockdown FOXA2 through misexpression of a targeted short hairpin RNA, DA neurons (TH+ cells) are severely reduced, consistent with the perturbation of MFP in dnFOXA2 overexpression experiments and as observed in foxa2+/− mice (Figs. 4.9M, N, red arrowheads, 4.4F; Ferri et al., 2007; Kittappa et al., 2007). It is also possible that the observed increase in DA neurons in FOXA2 and SHH electroporations is partially due to premature differentiation of the endogenous and ectopically induced DA progenitors, consistent with our previous results, which indentified a role for HH signaling in the differentiation of the ventral midbrain (Bayly et al., 2007). Intriguingly, when we analyzed the presence of DA neurons at E5.5, we found that both FOXA2 and SHH induced premature expression of TH by half a day (Fig. 4.9O, P). All together, these data suggest that DA progenitors originate from within the MFP and possess a later requirement for SHH to properly differentiate into mature TH+ neurons. These results also explain why HH blockade resulted in a depletion of TH expression, yet failed to affect the specification of the MFP (Figs. 3.1F, 4.2A).

**FOXA2 overexpression results in the premature differentiation of midbrain progenitors**

In order to further characterize the premature differentiation caused by misexpression of FOXA2, we attempted to identify the earliest time point that this
behavior could be observed. In order to test this, were compared cells ectopically expressing FOXA2 and compared their localization to cells which express NOTCH1. NOTCH1 mRNA is ubiquitously expressed in the progenitors of the midbrain neurepithelium as they go through the cell cycle (Higuchi et al., 1995). When midbrain progenitors exit the cell cycle, they stop the expression of NOTCH1, turn on expression of $\beta$-TUBULIN and migrate basally toward the pial surface of the neurepithelium (Sanders et al., 2002). Differentiated oculomotor neurons are the first cell type observed within the midbrain at E3, adjacent to the MFP (Sanders, 2001). When we electroporated EGFP, we observed that the electroporated cells were evenly distributed throughout the midbrain neurepithelium which was entirely composed of NOTCH1+ progenitors at E3 (Fig. 4.10A). Similarly, when we misexpressed FOXA2, we failed to observe a difference in the distribution of NOTCH1+ cells at E3 (Fig. 4.10B). However at E4, when EGFP-electroporated midbrains possessed only a small region of differentiated, NOTCH1- cells adjacent to the MFP, ectopic FOXA2 induced a comparatively dramatic increase in the amount of NOTCH1- cells suggesting a robust ability of FOXA2 to induce the premature differentiation of midbrain progenitors (Fig. 4.10C, D). Curiously, the observed increase in the prematurely differentiated cells (NOTCH1-) did not appear to come at the expense of progenitor cells (NOTCH1+), a phenotype normally associated with defective neurogenesis. A possible explanation is that FOXA2 simply accelerates the cell cycle, rather than altering the choice of progenitor cells to give rise to more neurons instead of additional progenitors. In support of this idea is the observation that the NOTCH1+ region of FOXA2-electroporated midbrains are of comparable size to those electroporated with EGFP, even though
Figure 4.10. FOXA2 overexpression results in the premature differentiation of midbrain progenitors

(A). Cells electroporated with *EGFP* (blue), are located entirely within the progenitor layer (*NOTCH1*, brown) at E3. (B). Similar to cells electroporated with *EGFP*, those electroporated with *FOXA2* (blue) are also located entirely within the *NOTCH1*+ progenitor layer (brown). Note, however, that a majority of the *FOXA2* + cells are concentrated along the pial surface. (C). At E4, cells electroporated with *EGFP* (blue) remain within the progenitor layer (*NOTCH1*, brown) and absent from the mantle layer (*NOTCH1*-, arrowhead). (D). Unlike cells electroporated with *EGFP*, the majority of those electroporated with *FOXA2* (blue), are found in an ectopically induced *NOTCH1*-region along the pial edge at E4 (arrowheads). Note the increased thickness of the midbrain neurepithelium after misexpression of *FOXA2* compared to *EGFP*-electroporated controls (double-headed arrows). *Abbreviations*: E: embryonic day; EP: electroporation; rFP: rostral floor plate.
FOXA2-electroporated midbrains possessed a significantly larger amount of differentiated, NOTCH- cells and were thicker in general.

**Differential regulation of cell proliferation and differentiation by FOXA2**

The role of HH signaling in regulating cell cycle progression and differentiation is highly context dependent (Bally-Cuif et al., 1995; Roy and Ingham, 2002; Masai et al., 2005; Neumann, 2005). Our previous studies have shown that HH signaling in the chick midbrain reduces cell proliferation and increases cell differentiation (Fig. 3.2; Bayly et al., 2007). This phenotype was mimicked by FOXA2 overexpression as a robust increase in *Class III β-TUBULIN* (a marker of newly differentiated neurons) was observed (Fig. 4.11A, B, arrows). The increase in *β-TUBULIN*+ cells was associated with a suppression of *CYCLIN D1* expression, but only a modest decrease in *NOTCH1* (Fig. 4.11A, B, arrowheads). Curiously, the reduced expression of midbrain progenitor markers occurred with only a minimal reduction of the ventricular layer. When we analyzed the differentiated cell types of the OMC (*ISL1*) and DA neurons (*LMX1B*) at E5, we found that the relative proportion of each cell type in the mantle layer was greatly increased and almost entirely made up the *β-TUBULIN*+ cell population (Fig. 4.11B, C). Additionally, we observed that the increase in differentiation by FOXA2 was dose-dependent. When we electroporated 0.1 mg/ml of FOXA2, ectopic *LMX1B* was induced sparingly and only found within the ventricular layer (n=3/3; Fig. 4.11D). When we increased the concentration to 0.5 mg/ml, the amount of ectopically induced *LMX1B* significantly increased and was observed within both the ventricular and mantle layer (n=3/3; Fig. 4.11E).
Figure 4.11. FOXA2 positively induces differentiation in a dose-dependent manner (A, B). Unilateral misexpression of FOXA2 results in the suppression of CYCLIND1 and NOTCH1 (blue, arrowhead, progenitor layer) and an increase in Class III β-Tubulin expression (brown, arrow, differentiated layer). Note, however, that the thickness of the ventricular layer is only marginally decreased. (C, D). The observed increase in differentiated cells types are predominantly made up of LMX1B+ cells (C, brown, DA) induced in a cell-autonomous fashion (EGFP, blue) and ISL1+ cells (D, brown, OMC) induced in a non-autonomous fashion. (E, F). FOXA2 induces differentiation in a concentration-dependent manner. At 0.1 mg/ml, FOXA2 is only sufficient to induce a small amount of LMX1B+ cells that remain in the ventricular layer (brown, MFP, arrowheads). However, at 0.5 mg/ml, FOXA2 is sufficient to increase the amount of LMX1B expression (brown) observed within the ventricular (arrowhead) and mantle layer (arrow). Note the change in morphology after electroporation of 0.5 mg/ml that is not observed with 0.1 mg/ml. Abbreviations: EP: electroporation.
Since FOXA2 is sufficient to induce ectopic differentiation and can do so prematurely, we reasoned that knocking down FOXA2 would conversely result in the retardation of the cell cycle such that cells containing less FOXA2 (containing the targeted RNAi vector) would remain in the ventricular layer longer than normal. Compared to control RNAi electroporations, FOXA2 RNAi caused a general reduction in the PHOX2A+ cells found in the mantle layer most likely due to a reduction in its transcriptional target, SHH (Fig. 4.12A, B, Fig. 4.7D). Curiously however, and unlike any HH perturbations, FOXA2 RNAi also led to the ectopic presence of PHOX2A+ cells within the ventricular layer, producing a small “bulge” along the pial surface similar to that observed after dnFOXA2 misexpression (Figs. 4.12B, arrowhead, 4.6G, H). To examine the cell autonomous role of FOXA2 RNAi in this particular cell behavior, we coelectroporated the RNAi vector with an EGFP plasmid. Consistent with a role in differentiation, the ectopic presence of PHOX2A in the ventricular layer was associated with EGFP+ cells, suggesting that FOXA2 may be necessary to exit the cell cycle or perhaps migrate basally after differentiation (Fig. 4.12C, D). Although a few EGFP+ cells were located in the mantle layer, it is possible that they did not possess the RNAi construct since coelectroporation does not always transfer both plasmids into a single cell (Brown and Agarwala, unpublished observations). However, when we examined cell cycle markers after the electroporation of FOXA2 RNAi, instead of observing an increase in ventricular markers at the expense of those of differentiation, we saw the opposite (Fig. 4.12E, F). This apparent reversal of polarity within the neurepithelium suggests that perhaps FOXA2 is most likely required for the basal migration of differentiated cells after they exit the cell cycle, rather than a general involvement in differentiation. In support of this idea, Foxa2
Figure 4.12. Knockdown of FOXA2 leads to a deregulation of epithelial morphology
(A, B). Compared to electroporated controls (A), embryos electroporated with FOXA2 RNAi showed a general reduction in the amount of PHOX2A+ cells (brown, OMC). Additionally, an ectopic group of PHOX2A+ cells were commonly observed to “bulge” out from the ventricular surface (arrowhead). (C, D). The ectopic “bulge” was always associated with cells electroporated with FOXA2 RNAi (blue, arrowhead). (E, F). Surprisingly, the ectopic “bulge” induced by FOXA2 RNAi was made up of differentiated, Class III β-Tubulin+ cells (brown, arrowhead, F), which were also CYCLIN D1- (blue).
is required to establish polarity and epithelialization during gastrulation in the mouse (Burtscher and Lickert, 2009).

**FOXA2 induces the formation of actively dividing rosettes in the mantle layer**

FOXA2 and SHH both positively regulate differentiation, suggesting that FOXA2 is likely acting through SHH to modulate the cell cycle. However, closer examination of FOXA2 misexpression reveals a more complex phenotype that was not seen after SHH electroporations. In addition to the increased differentiation and reduced proliferation, a handful of cells expressing midbrain neural progenitor markers (*NOTCH1, NGN2, MSX1, SHH, OTX2, MN CADHERIN*, and *CYCLIN D1*) were paradoxically observed in the mantle layer (Fig. 4.13A-G). Interestingly, these clusters of cells were actively undergoing cell division indicated by the incorporation of BrdU and were always associated with FOXA2 (Fig. 4.13F, H). Typically, but not ubiquitously, these cells were organized into “rosettes” which expressed increased levels of MN-Cadherin that surrounded a central lumen (Fig. 4.13F, I). The formation of similar rosette-like structures in the neurepithelium has been observed following BMP blockade, N-cadherin perturbations and after disruptions of apicobasal cell polarity (Eom et al, submitted; Ganzler-Odenthal and Redies, 1998; Klezovitch et al., 2004). Although we currently do not know the mechanism responsible for the formation of these “rosettes,” a detailed examination is currently ongoing (Eom et al., submitted). These data suggest that FOXA2 regulates the epithelial organization of the midbrain along the ventricular-pial (apicobasal) axis independently of SHH.
Figure 4.13. FOXA2 induces the formation of actively dividing rosettes in the mantle layer (A-G). Unlike SHH electroporations, overexpression of FOXA2 commonly resulted in the presence of ectopic clusters of cells found within the mantle layer that expressed markers normally restricted to the ventricular layer. These rosettes expressed NOTCH1 (blue, A), NGN2 (blue, B), MSX1 (blue, C), SHH (blue, D), CYCLIN D1 (blue, E), MN-Cadherin (blue, F) and OTX2 (brown, G) and were always associated with electroporated cells (G, arrowheads). (H). Additionally, these rosettes incorporated BrdU (brown, arrowhead), suggesting that cells within these rosettes were actively dividing. (I). An optical section using confocal microscopy reveals that the cells within a rosette surround a central lumen (arrowhead).
**Misexpression of GLI1 is sufficient to modestly induce MFP**

Interestingly, *FOXA2* can be induced by ectopic *SHH* throughout the midbrain, but curiously is never associated with an MFP fate (Fig. 4.14A; Agarwala and Ragsdale, 2002). A possible explanation for this observation is the potential lack of subcellular machinery required to activate target gene transcription. To circumvent the potential absence of effector molecules that may lie upstream to the transcriptional activity of the GLI proteins, we manipulated levels of GLI proteins directly and analyzed their ability to regulate *FOXA2* expression and to induce an MFP fate. As expected, overexpression of human *GLI1* led to the cell autonomous expression of *FOXA2* (Fig. 4.14B). Additionally, unlike *SHH* electroporations, overexpression of *hGLI1* (≤ 4 mg/ml) was sufficient to induce ectopic *LMX1B* although only at very low levels compared to *FOXA2* (1.2 mg/ml) (Fig. 4.14C, D). However, misexpression of *hGLI1* did not affect the number of DA neurons (*TH*+ cells), a cell-type originating from the MFP region of the midbrain, suggesting that the complete MFP program was not induced (Fig. 4.9, data not shown). Taken together, these data suggest that although SHH and GLI proteins can activate *FOXA2* expression and *hGLI1* overexpression can weakly induce MFP markers, the HH signaling pathway alone cannot account for the robust induction of the MFP by *FOXA2* and that other signaling molecules must also take part.

**FOXA2 and SHH differentially regulate the expression of BMP modulators**

The similarities observed between *FOXA2* overexpression and perturbations of BMP signaling in the regulation of epithelial morphology led us to question whether they may cooperate in the specification of the MFP (Figs. 4.6, 4.13; Eom et al., submitted). The cooperative action between SHH and levels of BMP is thought to be required in the
Figure 4.14. Misexpression of GLI1 is sufficient to modestly induce MFP
(A). SHH overexpression is sufficient to induce ectopic FOXA2 expression (blue) throughout ventral midbrain, but cannot induce MFP (see Fig. 4.3A, B; Agarwala and Ragsdale, 2002). (B). Overexpression of hGLI1 leads to the induction of FOXA2 (brown) in a cell autonomous manner (blue, arrowheads). (C, D). Overexpression of hGLI1 weakly induces MFP (C, LMX1B, brown, arrowheads), compared to the dramatic induction of LMX1B (D, brown) observed after misexpression of FOXA2 (D, blue). Abbreviations: EP: electroporation.
specification of hypothalamic DA neurons of the rostral diencephalic ventral midline (RDVM) and in the specification of the MFP of the spinal cord (Dale et al., 1997; Charrier et al., 2002; Ohyama et al., 2005). Interestingly, BMP7 is coexpressed with SHH in the rFP from H&H 8-9, but remains restricted to the MFP during later stages of development (Figs. 4.15A, B, 4.1E, data not shown; Fogel, 2009). Additionally, BMP5, BMP6 and MSX1, a transcriptional target of active BMP signaling, as well as secreted BMP antagonists NOGGIN and CHORDIN are expressed within the MFP (Fig. 4.4C, 4.15E; Fogel, 2009). Thus, many modulators of BMP signaling are properly positioned temporally and spatially to play a significant role in the development of the MFP. Similar to other genes whose expression is restricted to the MFP, FOXA2 robustly induced BMP7 expression throughout ventral midbrain while SHH could only do so in a regionally-restricted manner (Fig. 4.15C, D, compare to 4.16B). Additionally, the ectopic BMP7 induced by FOXA2 was associated with MSX1, which was not observed after SHH electroporations (Fig. 4.15E, F). Together, these results suggest that the ability of FOXA2 to induce an MFP fate may be through its regulation of BMP signaling, which SHH alone cannot do. Remarkably, this may also explain why SHH is able to convert the MHB into an MFP fate as BMP7 is present along both the MFP and the MHB, possibly acting as a cofactor for SHH to induce MFP.

**FOXA2 positively regulates the expression of LMX1B and BMPs independently of SHH**

Although FOXA2 is sufficient to induce an MFP fate throughout the ventral midbrain, it is also a robust transcriptional activator of SHH expression (Figs. 4.4A, 4.18D; Sasaki and Hogan, 1994). In order to analyze the effects of FOXA2 in the absence of SHH, we
Figure 4.15. FOXA2 and SHH differentially regulate the expression of BMP modulators

(A). BMP7 (blue), is coexpressed with SHH (brown) within the rFP at H&H 10. (B). At E5, BMP7 expression (blue) is restricted to the MFP, whereas SHH (brown) is expressed throughout MFP and LFP (Fogel, 2009). (C). Like other genes expressed within the MFP, misexpression of FOXA2 is sufficient to induce BMP7 (blue) throughout ventral midbrain. (D). In contrast, bilateral electroporation of SHH is only sufficient to induce BMP7 expression (blue) along the MHB (compare to B). (E). Additionally, FOXA2 overexpression (brown) leads to the induction of MSX1 (blue), a transcriptional target of BMP signaling, in a cell-autonomous manner (arrowheads). (F). Overexpression of SHH (brown), however, fails to upregulate MSX1 expression (blue).

coelectroporated FOXA2 and Ptc1\textsuperscript{loop2}. Theoretically, this enabled us to examine the function of ectopic FOXA2 in cells that are “blind” to any extracellular HH ligand. Using this paradigm, we first asked if FOXA2 was sufficient to induce ectopic MFP in the absence of HH and whether it was associated with increased expression of BMP signaling components. Notably, after the coelectroporation of FOXA2 and Ptc1\textsuperscript{loop2}, ectopic LMX1B expression was observed in regions lacking SHH expression and was induced cell autonomously, although not in all areas that were electroporated (Figs. 4.16A-C 3.1B). However, the LMX1B expression induced in the absence of HH signaling was also associated with ectopic hinge-points, further supporting a HH-independent role for FOXA2 in regulating epithelial morphology (Fig. 4.16D). Additionally, we found that FOXA2 misexpression caused the upregulation of BMP7 and MSX1 in the absence of HH signaling (Fig. 4.16E-H). These data suggest quite strikingly, that FOXA2 is sufficient to regulate the gene expression necessary for the specification of the MFP independently of HH - similar to the zebrafish model of floor plate development and in contrast to the mouse (Placzek and Briscoe, 2005).

**SHH functions as a switch between MFP and roof plate specification**

FOXA2 is sufficient to induce markers of the MFP in the absence of HH signaling, however, it remained unclear how SHH was involved in MFP specification as it is ubiquitously expressed within the MFP throughout its development. A closer examination of the results obtained by coelectroporations of FOXA2 and Ptc1\textsuperscript{loop2} appeared to reveal a mixed phenotype. Although FOXA2 ectopically induced the MFP
Figure 4.16. FOXA2 positively regulates the expression of LMX1B and BMPs independently of SHH (A, B). Misexpression of FOXA2 is sufficient to induce the MFP marker LMX1B (blue, arrowhead) in some regions lacking HH signaling (SHH- cells), but not all regions. (C). The induction of MFP (LMX1B, brown) by FOXA2 occurs cell-autonomously in the absence of HH signaling (EGFP, blue, arrow, arrowhead). (D). A cross-section through C reveals an increase in the number of differentiated LMX1B+ cells (arrowhead) in addition to the ventricular layer (arrow). Note the presence of an ectopically induced hinge-point at the site of LMX1B expression in the absence of HH signaling (arrow). (E, F). In the absence of HH signaling (SHH- cells), FOXA2 is sufficient to induce BMP7 expression (blue, arrowhead). (G, H). Similar to BMP7, electroporation of FOXA2 is sufficient to induce MSX1 (blue, arrowhead) in the absence of HH signaling.
markers $LMX1B$, $BMP7$, and $MSX1$ in the absence of HH signaling, they lacked $SHH$ expression. Thus, by definition, an ectopic MFP cell type was not induced and perhaps what we observed was a cell type with mixed gene expression profiles. However, an alternate and more parsimonious explanation could be that due to the presence of $Ptc1^{\Delta loop2}$ alone, we were simply observing a dorsalization of the ventral midbrain. Quite intriguingly, $LMX1B$, $BMP7$, and $MSX1$ are expressed in the dorsal (tectal) midbrain in addition to the MFP. $LMX1B$ expression is restricted to the roof plate, the dorsal most cell type, while $BMP7$ and $MSX1$ occupy increasingly broader territories (Fogel, 2009). Thus, to determine whether the induction of $LMX1B$ in the absence of HH signaling was due to the presence of $FOXA2$ or rather HH blockade, we compared misexpression of $Ptc1^{\Delta loop2}$ alone to coelectroporations of $FOXA2$ and $Ptc1^{\Delta loop2}$ with respect to the induction of dorsal cell types. In order to assay the extent to which misexpression of $Ptc1^{\Delta loop2}$ was able to induce dorsal cell types in the presence or absence of $FOXA2$, we analyzed expression of $GLI2$, $PAX7$ and $GDF7$. The $GLI2$ expression territory normally encompasses the entire dorsal midbrain except for the roof plate and extends ventrally to border the lateral edge of the $FOXA2$ expression domain (Aglyamova and Agarwala, 2007). $PAX7$ expression is also observed throughout the dorsal midbrain, except for the roof plate, but does not extend as far ventrally as does $GLI2$. $GDF7$ expression, by contrast, is wholly restricted to the roof plate and is coexpressed with $LMX1B$ (Lee et al., 1998). All together, $GLI2$, $PAX7$ and $GDF7$ expression represents the entire dorsal midbrain. Interestingly, in either the presence or absence of $FOXA2$, $Ptc1^{\Delta loop2}$ caused the induction of dorsal cell types ($GLI2^+$ or $PAX7^+$ cells), which was concomitant with the absence of $SHH$ expression (Fig. 4.17A-D). In contrast, $FOXA2$ and $Ptc1^{\Delta loop2}$ coelectroporations induced $GDF7$ expression, the most dorsal cell type, throughout the
Figure 4.17. SHH functions as a switch between MFP and roof plate specification (A-D). Coelectroporation of FOXA2 and Ptc1Δloop2 in addition to Ptc1Δloop2 electroporations alone are sufficient to induce both GLI2 (A, B, blue) and PAX7 (B, C, blue). (E). Surprisingly, FOXA2 overexpression was sufficient to induce GDF7 (blue, arrowheads), normally expressed in the roof plate, throughout ventral midbrain. (F). Curiously, HH blockade alone was only sufficient to induce ectopic GDF7 expression (blue) along the MFP (arrows) and the MHB (arrowhead), despite effectively disrupting the specification of the LFP. (G-I). Coelectroporation of FOXA2 and Ptc1Δloop2 caused induction of LMX1B (brown, G, arrowheads) throughout ventral midbrain, a large majority of which coexpressed GDF7 (blue, H, arrowheads) and were associated with electroporated cells (GFP, green, I). (J-L). In contrast, Ptc1Δloop2 electroporations alone did not lead to an increase in LMX1B expression (brown, J) and ectopic GDF7 (blue, K) was only observed along the MFP (arrow) and MHB (arrowhead) despite a robust bilateral electroporation through ventral midbrain (GFP, green, asterisk, L).

ventral midbrain, whereas with $Ptc1^{\Delta\text{loop}2}$ alone, $GDF7$ was only observed along the medial region of floor plate and the MHB (Fig. 4.17E, F). The restricted induction of $GDF7$ was surprising, given the massive bilateral electroporation of $Ptc1^{\Delta\text{loop}2}$ that was sufficient to prevent $SHH$ expression in the LFP (Fig. 4.17F). Because $GDF7$ is coexpressed with $LMX1B$ in the roof plate, which is also present in the MFP and the MHB, we hypothesized that the $GDF7$ induced by $FOXA2$ and $Ptc1^{\Delta\text{loop}2}$ and $Ptc1^{\Delta\text{loop}2}$ electroporations only occurred where $LMX1B$ expression was present. In the absence of HH signaling, $FOXA2$ was sufficient to induce $LMX1B$ expression throughout the ventral midbrain some of which coexpressed $GDF7$ (Fig. 4.17G-I, arrowheads). Conversely, ubiquitous misexpression of $Ptc1^{\Delta\text{loop}2}$ only elicited ectopic $GDF7$ expression in $LMX1B^+$ cells located within the MFP and along the MHB (Fig. 4.17J-L). Together, these data quite convincingly suggest that $FOXA2$ is sufficient to induce $LMX1B$ expression in the absence of HH signaling, but also that SHH functions in the ventralization of the MFP. This conclusion also explains why we observed ectopic $LMX1B$ in regions which lacked $SHH$ expression in $FOXA2$ and $Ptc1^{\Delta\text{loop}2}$ coelectroporations; the MFP fate that would have normally been induced by $FOXA2$ alone was converted to a roof plate fate in the absence of HH signaling. Strikingly, these data also suggest a potent overarching role for $LMX1B$ in the establishment of signaling centers whereby the presence or absence of a single molecule can switch cell fates that normally occupy opposite ends of the dorsal/ventral cell-fate spectrum.
Figure 4.18. Supplementary Figures

(A, B). Magnified views of the ventral midline of the midbrain in H&H 10 embryos, where anterior is to the right. (A). Mosaic expression of FOXA2 (brown) and SHH (blue) is observed within the floor plate displaying areas of low (arrowhead) and high (arrow) expression. (B). Mosaic expression of LMX1B (blue) is also observed within the floor plate (arrow, arrowhead). (C). FOXA2 expression (brown) is observed within a group of differentiated cells subjacent to the MFP (arrow) in addition to a group of cells lateral to the FP (arrowhead). (D). Misexpression of FOXA2 (EGFP, blue) causes the induction of SHH (brown, arrowheads). (E, F). Misexpression of FOXA2 induces CHORDIN expression (blue, arrowheads), although is improperly localized to the pial surface of the neurepithelium (F, arrowheads) compared to CHORDIN expression observed within the MFP (arrow). (G). Misexpression of dnFOXA2 suppresses SHH expression (brown) within the MFP (arrowheads). Abbreviations: A: anterior; E: embryonic day; EP: electroporation; H&H: Hamburger and Hamilton Stage; MHB: midbrain-hindbrain boundary; P: posterior.
DISCUSSION

In this study, we focused on the contributions of both HH and FOXA2 activity in the context of midbrain FP development. We show that the midbrain FP can be partitioned into medial (MFP) and lateral (LFP) regions on the basis of gene expression and mode of induction. We provide evidence that HH signaling is necessary and sufficient for the specification of the LFP, but not the MFP after H&H 5. In contrast, FOXA2 alone can induce an MFP fate including the cell-shape changes associated with this cell type. Additionally, misexpression of FOXA2 causes a robust increase in both DA progenitors and neurons that was not observed after misexpression of SHH. We also demonstrate that FOXA2 can activate the expression of BMP7 and MSX1 in an HH-independent fashion and that SHH is required early as a ventralization signal. We suggest that FOXA2-mediated induction of BMP signaling may provide a mechanism which underlies the functional differences observed between FOXA2 and SHH and thus the specification of MFP and LFP.

Midbrain floor plate can be partitioned into medial and lateral territories on the basis of gene expression

Previous studies have suggested that the FP at spinal cord levels can be divided into MFP and LFP regions on the basis of gene expression, mode of induction and embryonic origin, however the mechanisms of specification and the organization of the FP in more rostral regions of the neural tube have not yet been examined in detail (Odenthal et al., 2000; Charrier et al., 2002). Gene expression analyses reveal that both SHH and FOXA2 appear along the ventral midline of the presumptive midbrain at H&H.
4+ (Ruiz i Altaba et al., 1995; Aglyamova and Agarwala, 2007). Until approximately H&H 15, \textit{SHH}, \textit{FOX}A2, \textit{LMX}1B, and \textit{BMP}7 are coexpressed in the rFP (Figure 1). After H&H 15, the expression territories of both \textit{SHH} and \textit{FOX}A2 extend laterally, while \textit{LMX}1B and \textit{BMP}7 remain restricted medially. This division of FP into medial and lateral regions continues through development until at least E6 where we have operationally defined MFP as (\textit{SHH}+, \textit{FOX}A2+, \textit{LMX}1B+, \textit{BMP}7+, \textit{DISP}1+) and LFP as (\textit{SHH}+, \textit{FOX}A2+, \textit{LMX}1B-, \textit{BMP}7-, \textit{DISP}1-). Interestingly, at approximately E4, a subset of cells within the MFP located along the ventral midline begins to express \textit{CHD}, \textit{BMP}5, and \textit{TAL}1 (Fig. 4.1F, Fogel, 2009, data not shown). The differential gene expression observed suggests an even further partitioning of midbrain FP, however, an exclusive mechanism of specification or separate function for this region of tissue has not been identified.

MFP and LFP also display differential gene expression patterns in the chick spinal cord where MFP is identified by the expression of \textit{FOX}A2 and the absence of \textit{SOX}1 while \textit{SOX}1 and transient expression of \textit{FOX}A2 are present in the LFP (Charrier et al., 2002). The lack of \textit{SOX}1 expression, a neural plate marker, in the MFP of spinal cord is due to the fact that these cells are node-derived and mesendodermal in origin (Catala et al., 1996; Charrier et al., 2002). Unlike the spinal cord, the midbrain is entirely \textit{SOX}2 positive (data not shown), suggesting that cells from the prechordal plate, node, or the notochord do not constitute any part of midbrain FP and that rFP is specified by a separate mechanism (Rex et al., 1997).
Midbrain floor plate can be partitioned into medial and lateral territories on the basis of mode of induction

We have previously shown through HH blockade experiments and the examination of the talpid2−/− phenotype, a HH pathway mutant, that HH signaling is not necessary after H&H 5 for the specification of the medial region of midbrain FP (Agarwala et al., 2005; Bayly et al., 2007). Examined in further detail, we have found that HH blockade is unable to affect the expression of either LMX1B, DISP1, or BMP7—all of which are expressed in our operationally defined MFP (Fig. 4.2, data not shown). Similarly in zebrafish smoothened mutants, the absence of HH signaling prevents the specification spinal cord LFP, but not the MFP (Chen et al., 2001). The inability of HH blockade to affect the specification of MFP is complemented by SHH overexpression experiments in which SHH robustly induces the LFP territory, yet can only specify MFP in a regionally-restricted manner along the MHB (Fig. 4.3). Our observations are supported by experiments in the spinal cord in which transplantations of SHH-expressing cells or LFP tissue onto a host neural tube induced markers of LFP, but could not induce gene expression that resembled that of the MFP (Charrier et al., 2002). However, transplantations of spinal cord MFP or notochord were sufficient to elicit such a pattern, suggesting that signal(s) in addition to SHH are secreted from the notochord to properly specify MFP (Charrier et al., 2002).

In zebrafish, the coordinated efforts of both Nodal and Shh signaling from the notochord are required for the full specification of the MFP (Strahle et al., 2004). In addition, in chick it has been suggested that NODAL and SHH synergistically act early in development to induce a group of cells anterior to Henson’s node in the epiblast, termed ‘area a,’ to acquire an FP fate that eventually migrate caudally and incorporate into
anterior FP (Schoenwolf and Sheard, 1990; Patten et al., 2003). Thus, NODAL seemed poised as an ideal candidate to work in cooperation with SHH in the specification of the MFP fate in ventral midbrain. However, when we manipulated NODAL signaling through overexpression of Nodal-related 2 (Cyclops), a constitutively active form of Smad2, a downstream effector of Nodal signaling, or by misexpression of a dominant negative form of Fast1, an obligate effector, they each failed to affect midbrain patterning (data not shown; Rebagliati et al., 1998; Muller et al., 1999; Muller et al., 2000). Interestingly, Foxa2, a known target of nodal signaling in zebrafish, is sufficient to rescue the absence of FP in these mutants suggesting that the regulation of foxa2 is critical for the proper specification of FP (Rastegar et al., 2002).

In monorail/foxa2 zebrafish mutants where foxa2 function is disrupted, FP is initially specified, but fails to fully differentiate and cannot be maintained (Norton et al., 2005). Whereas in mouse, foxa2 mutants fail to properly form a node and notochord, resulting in the complete absence of FP (Ang and Rossant, 1994; Weinstein et al., 1994). Because of its early requirement in development, understanding foxa2 function in FP specification has been difficult. Conditional overexpression of FOXA2 in mouse revealed its potency in inducing FP, but whether or not FOXA2 differentially affected MFP and LFP was not examined (Sasaki and Hogan, 1994). We have found that misexpression of FOXA2 in presumptive chick midbrain is sufficient to induce the entire FP. Cell-autonomously, FOXA2 upregulates markers of MFP (LMX1B+, DISP1+, BMP7+, and SHH+) and the induced secretion of SHH in turn specifies LFP (LMX1B-, DISP1-, BMP7-, and SHH+) cell-nonaутonomously (Fig. 4.4). This is in sharp contrast to SHH overexpression whose ability to induce MFP markers is restricted to an area just anterior to the MHB (Fig. 4.3). Misexpressing a dominant-negative form of FOXA2
prevents the expression of all rFP markers, whereas HH blockade can only disrupt the specification of LFP. Together these data suggest that although SHH and FOXA2 activate the expression of each other, FOXA2 is necessary and sufficient for the specification of the MFP and that its initial induction occurs independently of HH signaling. It remains to be seen what signaling molecules initiate FOXA2 expression or its activation in the neurepithelium, however, preliminary evidence suggests that it is BMP-mediated (data not shown). It also cannot be ruled out that the initial population of ventral midline cells that become the midbrain MFP emigrate from other tissues such as ‘area a’ or the rostral diencephalic ventral midline (RDVM; Dale et al., 1997).

**FOXA2 regulates the expression of BMP modulators independently of HH signaling**

The differential competence between FOXA2 and SHH overexpression to induce an MFP fate led us to look for additional genes that FOXA2 may be targeting that could work in cooperation with SHH to specify MFP. The cooperation between SHH and additional signaling molecules in the specification of ventral cell types has previously been reported as a mechanism in several organisms along different regions of the anteroposterior axis of the neural tube (Placzek and Briscoe, 2005). In chick, the observed inability of SHH to specify the MFP fate along the spinal cord is thought to occur because of an additional requirement such as BMP antagonists like Chordin (Charrier et al., 2002; Patten et al., 2003). However, for the specification of hypothalamic DA neurons and the RDVM, SHH requires the cooperative action of BMP7 (Dale et al., 1997; Ohyama et al., 2005). BMP7 is coexpressed with SHH and LMX1B
early in the development of rFP (H&H 8-9) and remains restricted to the MFP during later stages of development, and thus is properly positioned temporally and spatially to play a significant role in MFP development. Like other genes whose expression is restricted to the MFP, FOXA2 robustly induced BMP7 expression throughout ventral midbrain while SHH could only do so along the MHB. Interestingly, BMP7 is also expressed along the MHB, suggesting the possibility that SHH may require the modulation of BMP signaling to specify an MFP fate. In support these observations, overexpression of the BMP antagonist, NOGGIN, caused defects in epithelial morphology similar to those observed after misexpression of FOXA2, including ectopic hinge-points and the formation of “rosettes,” that were not observed after electroporation of SHH alone (Fig. 4.13; Eom et al., submitted). Interestingly, the ability of FOXA2 to induce BMP7 and its transcriptional target, MSX1, in the absence of HH signaling suggests that FOXA2 is upstream of HH and regulates additional pathways such as BMP signaling. Taken together, our results suggest that the specification and morphology of the MFP requires the coordinated efforts of both BMP and HH signaling, explaining the ability of FOXA2 to generate a fully competent MFP, whereas SHH alone cannot.

The medial floor plate is required for the proper morphology of the midbrain

The sufficiency of FOXA2, but not SHH, to specify MFP throughout ventral midbrain allowed us to examine its function as a cell-type in the developing midbrain by contrasting FOXA2 and SHH overexpression. One of the most apparent differences we observed between FOXA2 and SHH overexpression was the severe morphological
changes FOXA2 elicited. Cells in close proximity to those that received ectopic FOXA2 produced a bending or hinging of the tissue (Fig. 4.6). At the ectopic hinge-point, cells possessed basally located nuclei and an enrichment of apically localized F-actin, each of which are observed during the process of apical constriction (Schoenwolf and Smith, 2000). The first step during neurulation involves the folding of the neurepithelium at the midline of the embryo. This morphological process occurs in part due to changes in cell-shape of specific cells that are positioned along the midline. Originally cuboidal in shape, these cells become constricted at their apical surface and their nuclei translocate basally, ultimately causing a change to a wedge-shaped morphology. As a group, these wedge-shaped cells effectively create a hinge-point forcing the lateral regions of the neurepithelial sheet closer together (Schoenwolf and Smith, 2000). Interestingly, the wedge-shaped cells of the chick spinal cord express markers of MFP, but not LFP (Charrier et al., 2002). Similarly in the midbrain, we see that after misexpression of FOXA2, the MFP marker, *LMX1B* is localized to cells at the hinge-point of ectopic tissue bending (Fig. 4.6A, B). These data suggest that the initial bending of the neurepithelium during neurulation is one of the critical functions of the MFP. To our knowledge, this is the first example demonstrating the ability of a single transcription factor to regulate such a critical cell behavior. FOXA2 and its transcriptional targets therefore become potential candidates in studying epithelial cytoarchitecture and neurulation. Although we are uncertain if *LMX1B* is a direct target of FOXA2, its gene expression pattern suggests that it plays a large role in regulating morphological changes. Early in development, *LMX1B* is expressed in the isthmus and the dorsal lateral hinge-points in addition to the floor plate, each of which are critically important in shaping the midbrain (Fig. 4.6A, B; Yuan and Schoenwolf, 1999). However, very little is known about how *LMX1B* is
regulated or how it is functioning to shape tissue in the rFP and is currently under investigation in our laboratory.

The medial floor plate gives rise to dopaminergic neurons

An equally important difference observed between FOXA2 and SHH misexpression is the ability of FOXA2 to robustly induce DA neurons whereas SHH alone cannot. This is largely due to the widespread induction of both LMX1A and LMX1B by FOXA2 but not SHH (Fig. 4.9A-D). The ectopic expression of LMX1A and LMX1B caused by FOXA2 is accompanied by NURR1 and PITX3, all of which have been implicated in the specification and differentiation of DA neurons (Zetterstrom et al., 1997; Nunes et al., 2003; Maxwell et al., 2005; Andersson et al., 2006). Conversely, RNAi-mediated knockdown of FOXA2 leads to a reduction of TH. Likewise in mouse, overexpression of Foxa2 induces ectopic TH expression while reducing the dosage of Foxa2 leads to a reduction in the number of DA neurons (Sasaki and Hogan, 1994; Ferri et al., 2007; Kittappa et al., 2007). The restricted expression of LMX1A, LMX1B, and NURR1 to the MFP suggests that the large majority of DA progenitors are initially specified in the MFP of the midbrain. This also appears to be the case in mouse where lineage analysis has shown that a large proportion of cells originating from a very medial region of midbrain floor plate give rise to DA neurons (Ono et al., 2007). Interestingly, although SHH alone was insufficient to induce markers of DA progenitors, it was capable of marginally increasing the amount of TH+ positive cells (Fig. 4.9K). The observed increase in TH+ cells induced by ectopic SHH expression is likely to be the consequence of DA progenitors induced along the MHB (MFP) in addition to the premature
differentiation of the endogenous population of DA progenitors (Fig. 4.9C, P). A role for 
SHH alone in the differentiation of DA progenitors rather than in their induction is also 
consistent with our previous findings in which we reported that in the absence of HH 
signaling, DA progenitors (LMX1B+ cells) were still specified, yet DA neurons (TH+ cells) 
were severely reduced (Fig. 4.2A, B; Bayly et al., 2007). We propose that SHH provides 
a permissive environment for the induction of DA progenitors (Fig. 4.17) and is required 
later for their differentiation similar to what has been proposed for motor neurons of the 
spinal cord (Ericson et al., 1996).
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Chapter 5: Discussion

In this study, we have aimed to identify the molecular mechanisms necessary for the development of midbrain floor plate and to analyze how the floor plate functions in cell-fate specification and neurulation. Specifically, we provide evidence that midbrain floor plate is divided into MFP and LFP regions on the basis of gene expression and mode of induction and function. Interestingly, in vivo misexpression of \textit{Ptc1\textsuperscript{4loop2}} and mutant analyses of the chick \textit{talpid\textsuperscript{2}} have suggested that MFP, but not LFP can develop in the absence of HH signaling (Agarwala et al., 2005; Bayly et al., 2007). Although properly specified, the remaining ventral cell types are scattered and lack cohesive organization (Agarwala et al., 2005; Bayly et al., 2007). Conversely, misexpression of \textit{SHH} is only sufficient to induce MFP along the midbrain-hindbrain boundary suggesting that MFP requires additional signals for its specification that LFP does not, similar to that observed in zebrafish \textit{smu and syu} mutants defective in HH signaling (Schauerte et al., 1998; Chen et al., 2001; Karlstrom et al., 2003). However, we show that misexpression of \textit{FOXA2} in the midbrain is sufficient to initiate the entire midbrain pattern, including genes expressed in MFP (\textit{BMP7}, \textit{DISP1}, \textit{LMX1B}) that cannot be induced by SHH alone. The ectopic induction of MFP is associated with a robust increase in dopaminergic neurons that is not observed after \textit{SHH} misexpression. The MFP induced by \textit{FOXA2} misexpression displays an altered tissue morphology resulting in ectopic hinging or bending of the neural tube, which mirror that of the endogenous MFP and can occur in the absence of HH signaling. Additionally, we provide evidence that FOXA2, but not SHH, can regulate modulators of BMP signaling, which may underlie their functional differences. Taken together, our data sheds new light into the mechanism by which...
midbrain floor plate is specified and ascribes critically important functional roles to both the MFP and the LFP.

**Midbrain floor plate can be partitioned into medial and lateral territories on the basis of gene expression**

FP is best characterized in zebrafish, where gene expression and mutant analyses have revealed a partitioning of FP into medial and lateral regions (Odenthal et al., 2000). In zebrafish, the three- to four-cell wide spinal cord FP is made up of a single row of cells along the ventral midline (MFP) and a row of cells that flank both sides of the MFP (LFP), which differ greatly in their gene expression profiles. *shh, tiggy-winkle hedgehog, collagen2A1, netrin-1, fkd7* (Hnf3α homolog), *fkd4* (Xenopus *Pintallavis* homolog), *f-spondin*, and *axial* (Hnf3β/Foxa2 homolog) are all expressed within the MFP, while *axial* and *fkd4* are expressed in both the MFP and LFP (Krauss et al., 1993; Strahle et al., 1993; Ekker et al., 1995; Yan et al., 1995; Strahle et al., 1997a; Odenthal and Nusslein-Volhard, 1998). However, *nkx2.2b* is the only gene that has been identified, which is exclusively expressed within the LFP (Schafer et al., 2005).

In mouse, gene expression in the midbrain and spinal cord also suggests a partitioning of FP into medial and lateral regions (Placzek et al., 1993; Roelink et al., 1995; Kittappa et al., 2007; Fogel et al., 2008). However, both divisions of the FP appear to be dependent upon HH signaling (Chiang et al., 1996; Zhang et al., 2001b; Fogel et al., 2008).

As in zebrafish and mouse, the FP in the chick spinal cord can be divided into MFP and LFP based on differential gene expression patterns. MFP is identified by the
expression of FOXA2 and the absence of SOX1 while SOX1 and transient expression of FOXA2 are present in the LFP (Charrier et al., 2002).

Our characterization of gene expression in the developing rFP has revealed that both SHH and FOXA2 appear along the ventral midline of the presumptive midbrain at H&H 4+ (data not shown; Ruiz i Altaba et al., 1995; Aglyamova and Agarwala, 2007). Until approximately H&H 15, SHH, FOXA2, LMX1B, and BMP7 are coexpressed in the rFP (Figure 1). After H&H 15, the expression territories of both SHH and FOXA2 extend laterally, while LMX1B and BMP7 remain restricted medially. This division of FP into medial and lateral regions continues through development until at least E6 where we have operationally defined MFP as (SHH+, FOXA2+, LMX1B+, BMP7+, DISP1+) and LFP as (SHH+, FOXA2+, LMX1B-, BMP7-, DISP1-). Interestingly, at approximately E4, a subset of cells within the MFP located along the ventral midline begins to express CHD, BMP5, and TAL1 (Fig. 4.1F; Fogel, 2009; data not shown). The differential gene expression observed suggests an even further partitioning of midbrain FP, however, an exclusive mechanism of specification or separate function for this region of tissue has not been identified.

Midbrain floor plate can be partitioned into medial and lateral territories based on differential modes of induction

Differences between SHH and FOXA2

We have previously shown through HH blockade experiments and the examination of the talpid2/− phenotype, a HH pathway mutant, that HH signaling is not necessary after H&H 5 for the specification of the medial region of midbrain FP.
(Agarwala et al., 2005; Bayly et al., 2007). Examined in further detail, we have found that HH blockade is unable to affect the expression of either *LMX1B*, *DISP1*, or *BMP7*—all of which are expressed in our operationally defined MFP (Fig. 4.2, data not shown). Similarly in zebrafish *smoothened* mutants, the absence of HH signaling prevents the specification spinal cord LFP, but not the MFP (Chen et al., 2001). The inability of HH blockade to affect the specification of MFP is complemented by *SHH* overexpression experiments in the chick midbrain where *SHH* robustly induces the LFP territory, yet can only specify MFP in a regionally-restricted manner along the MHB (Fig. 4.3). Our observations are supported by in vitro experiments where exogenously supplied SHH was unable to affect the amount of *LMX1B*+ cells and in the spinal cord in which transplantations of *SHH*-expressing cells or LFP tissue onto a host neural tube induced markers of LFP, but could not induce gene expression that resembled that of the MFP (Charrier et al., 2002; Kittappa et al., 2007). However, transplantations of spinal cord MFP or notochord were sufficient to elicit such a pattern, suggesting that signal(s) in addition to SHH are secreted from the notochord to properly specify MFP (Charrier et al., 2002).

In zebrafish, the coordinated efforts of both Nodal and Shh signaling from the notochord are required for the full specification of the MFP (Strahle et al., 2004). In addition, in chick it has been suggested that NODAL and SHH synergistically act early in development to induce a group of cells anterior to Henson’s node in the epiblast, termed ‘area a,’ to acquire an FP fate that eventually migrate caudally and incorporate into anterior FP (Schoenwolf and Sheard, 1990; Patten et al., 2003). Thus, NODAL seemed poised as an ideal candidate to work in cooperation with SHH in the specification of the MFP fate in ventral midbrain. However, when we manipulated NODAL signaling through
overexpression of *Nodal-related 2* (*Cyclops*), a constitutively active form of *Smad2*, a downstream effector of Nodal signaling, or by misexpression of a dominant negative form of *Fast1*, an obligate effector, they each failed to affect midbrain patterning (data not shown). Interestingly, Foxa2, a known target of nodal signaling in zebrafish, is sufficient to rescue the absence of FP in these mutants suggesting that the regulation of foxa2 is critical for the proper specification of FP (Rastegar et al., 2002).

In monorail/foxa2 zebrafish mutants where foxa2 function is disrupted, FP is initially specified, but fails to fully differentiate and cannot be maintained (Norton et al., 2005). Whereas in mouse, foxa2⁻/⁻ mutants fail to properly form a node and notochord, resulting in the complete absence of FP (Ang and Rossant, 1994; Weinstein et al., 1994). Because of its early requirement in development, understanding foxa2 function in FP specification has been difficult. Conditional overexpression of *FOXA2* in mouse revealed its potency in inducing FP, but whether or not *FOXA2* differentially affected MFP and LFP was not examined (Sasaki and Hogan, 1994). We have found that misexpression of *FOXA2* in presumptive chick midbrain is sufficient to induce the entire FP. Cell-autonomously, *FOXA2* upregulates markers of MFP (*LMX1B*, *DISP1*, *BMP7*, and *SHH*) and the induced secretion of SHH in turn specifies LFP (*LMX1B*, *DISP1*, *BMP7*, and *SHH*) cell-nonautonomously. This is in sharp contrast to *SHH* overexpression whose ability to induce MFP markers is restricted to an area just anterior to the MHB (Fig. 4.2). Misexpressing a dominant-negative form of *FOXA2* prevents the expression of all rFP markers, whereas HH blockade can only disrupt the specification of LFP. Together these data suggest that although SHH and FOXA2 activate the expression of each other, only FOXA2 is necessary and sufficient for the specification of
the MFP, however, it remains to be seen what signaling molecules initiate FOXA2 expression or its activation.

**FOXA2 regulates the expression of BMP modulators independently of HH signaling**

The differential competence between FOXA2 and SHH overexpression to induce an MFP fate led us to look for additional genes that FOXA2 may be targeting that could work in cooperation with SHH to specify MFP. The cooperation between SHH and additional signaling molecules in the specification of ventral cell types has previously been reported as a mechanism in several organisms along different regions of the anteroposterior axis of the neural tube (Placzek and Briscoe, 2005). In chick, the observed inability of SHH to specify the MFP fate along the spinal cord is thought to occur because of an additional requirement such as BMP antagonists like Chordin (Charrier et al., 2002; Patten et al., 2003). However, for the specification of hypothalamic DA neurons and the RDVM, SHH requires the cooperative action of BMP7 (Dale et al., 1997; Ohyama et al., 2005). At approximately HH 8-9, BMP7 is coexpressed with SHH and LMX1B in the rFP and remains restricted to the MFP during later stages of development, thus properly positioned temporally and spatially to play a significant role in MFP development (Fig. 4.15; Fogel, 2009). Like other genes whose expression is restricted to the MFP, FOXA2 robustly induced BMP7 expression throughout ventral midbrain while SHH could only do so along the MHB. Interestingly, BMP7 is also expressed along the MHB, suggesting the possibility that SHH may require the modulation of BMP signaling to specify an MFP fate as SHH overexpression is only
sufficient to induce an MFP fate along the MHB (Figs. 4.1E; 4.3). However, in our hands, gain- and loss-of-function experiments manipulating BMP signaling with or without SHH was never observed to induce or suppress ventral midbrain cell fates (data not shown; Eom et al., submitted; Fogel, 2009). Although gene expression was not altered, we have observed that BMP blockade causes defects in epithelial morphology similar to those observed after misexpression of FOXA2, including the formation of ectopic hinge-points and “rosettes,” that were not observed after electroporation of SHH alone (Fig. 4.13; Eom et al., submitted). Taken together, our results suggest that the ability of FOXA2, but not SHH, to greatly affected the morphology of the midbrain neurepithelium is likely due to the modulation of BMPs by FOXA2 (Fig. 4.15). However, the HH-independent modulation of BMP signaling appears not to be involved in the specification of the MFP (data not shown; Fogel, 2009).

Midbrain floor plate can be partitioned into medial and lateral territories based on functional differences

The medial floor plate is required for the proper morphology of the midbrain

The sufficiency of FOXA2, but not SHH, to specify MFP throughout ventral midbrain allowed us to examine the function of the MFP by contrasting the effects observed after FOXA2 and SHH overexpression. One of the most apparent differences we observed between FOXA2 and SHH was the severe morphological changes FOXA2 elicited. Cells in close proximity to those that received ectopic FOXA2 produced a bending or hinging of the tissue (Fig. 4.6). Overexpression of SHH, on the other hand, only led tissue bending along the MHB and an exaggeration of the endogenous median
hinge-point, consistent to what is observed in the spinal cord (data not shown; Charrier et al., 2002). At the ectopic hinge-point, cells possessed basally located nuclei and an enrichment of apically localized F-actin, each of which are observed during the process of apical constriction (Schoenwolf and Smith, 2000). Misexpression of *dnFOXA2* or FOXA2 RNAi, in contrast, induced “bulges” in the midbrain neurepithelium that appeared to bend the tissue in the opposite direction of the “hinges” observed after *FOXA2* overexpression (Fig. 4.6).

One of the first steps during neurulation involves the folding of the neurepithelium at the midline of the embryo. This morphological process occurs in part due to changes in cell-shape of specific cells that are positioned along the midline. Originally cuboidal in shape, these cells become constricted at their apical surface and their nuclei translocate basally, ultimately causing a change to a wedge-shaped morphology (Schoenwolf and Smith, 2000). As a group, these wedge-shaped cells effectively create a hinge-point forcing the lateral regions of the neurepithelial sheet closer together (Schoenwolf and Smith, 2000). Interestingly, the wedge-shaped cells of the chick spinal cord express markers of MFP, but not LFP (Charrier et al., 2002). Similarly in the midbrain, we see that after misexpression of *FOXA2*, the MFP marker, *LMX1B*, is localized to cells at the hinge-point of ectopic tissue bending (Fig. 4.6A, B). However, not all cells that ectopically express *LMX1B* form a hinge-point, possibly due to differing concentrations of ectopic *FOXA2* received by the cell after electroporation. These data suggest that the formation of the median hinge-point during neurulation is one of the critical functions of the MFP and not the LFP, as the LFP appears after neural tube closure.

In addition to inducing hinge-points, overexpression of *FOXA2* causes the formation of “rosettes,” located within the mantle layer of midbrain neurepithelium (Fig.
These rosettes display a neural progenitor phenotype (SHH+, NGN2+ and OTX2+), are undergoing cell division (BrdU+, Cyclin D1+), express elevated levels of cadherin (MN-Cadherin) and surround a central lumen. Intriguingly, “rosettes” are also observed after perturbations of BMPs, apicobasal determinants and N-Cadherin (Eom et al., submitted; Ganzler-Odenthal and Redies, 1998; Klezovitch et al., 2004; Fogel, 2009). The shared ability of FOXA2 and BMP blockade to deregulate the normal epithelial morphology suggests that FOXA2 may be acting through BMP signaling in these processes, consistent with the observed upregulation of BMP modulators after FOXA2 misexpression. Indeed, preliminary results from our laboratory reveal that FOXA2 directly interacts with phosphorylated forms of Smad1, 5, and 8, downstream targets of the canonical BMP signaling pathway (Raghavan et al., unpublished observations).

**The medial floor plate gives rise to dopaminergic neurons**

An equally important difference observed between FOXA2 and SHH misexpression is the ability of FOXA2 to robustly induce DA neurons whereas SHH alone cannot. This is largely in part due to the widespread induction of both LMX1A and LMX1B by FOXA2 and not SHH (Fig. 4.9A-D). We show that the ectopic expression of LMX1A and LMX1B caused by FOXA2 is accompanied by NURR1 and PITX3, all of which have been implicated in the specification and differentiation of DA neurons (Zetterstrom et al., 1997; Nunes et al., 2003; Maxwell et al., 2005; Andersson et al., 2006). Conversely, we demonstrate that RNAi-mediated knockdown of FOXA2 leads to a reduction of TH. Likewise in mouse, overexpression of Foxa2 induces ectopic TH
expression while reducing the dosage of Foxa2 leads to a reduction in the amount of DA neurons (Sasaki and Hogan, 1994; Ferri et al., 2007; Kittappa et al., 2007). The restricted expression of LMX1A, LMX1B, and NURR1 to the MFP suggests that the large majority of DA progenitors are initially specified in the MFP of the midbrain. This also appears to be the case in mouse where lineage analysis has shown that a large proportion of cells that originate from a medial region of midbrain floor plate give rise to DA neurons (Ono et al., 2007). Interestingly, although SHH alone was insufficient to induce markers of DA progenitors away from the MHB, it was capable of increasing the number of TH+ positive cells (Fig. 4.9K). The observed increase in TH+ cells induced by ectopic SHH expression is likely to be the consequence of DA progenitors induced along the MHB (MFP; Figs. 3.3A, B; 4.9C) in addition to the premature differentiation of DA progenitors (Fig. 4.9P).

A role for SHH alone in the differentiation of DA progenitors rather than in their induction is also consistent with our previous findings in which we reported that in the absence of HH signaling, DA progenitors (LMX1B+ cells) were still specified, yet DA neurons (TH+ cells) were severely reduced (Fig. 4.2A, B; Bayly et al., 2007). In dissociated cells derived from E8.5 mouse midbrains, exogenous SHH was unable to induce dopaminergic progenitors (LMX1B+ cells) and FOXA2 was able to induce TH+ cells in the presence of cyclopamine (Kittappa et al., 2007). We propose that SHH provides a permissive environment for the induction of DA progenitors early in development (Fig. 4.17) and is required later for their differentiation, similar to what has been proposed for motor neurons of the spinal cord (Ericson et al., 1996).
Lateral floor plate is required for the proper organization of ventral midbrain cell fates and the maintenance of its regional boundaries

In order to examine the functions of the lateral floor plate, we analyzed the phenotypes elicited by HH blockade as in these perturbations, the LFP is disrupted, while the MFP remains intact (Fig. 4.2). One of the most severe phenotypes observed after misexpression of Ptc1\textsuperscript{∆loop2} was increased cell-scatter and a disruption of the arc pattern (Fig. 3.1; Fig. 3.4E). Additionally, a loss of spatial organization has also been reported in several HH pathway mouse mutants (Shh:Gli3\textsuperscript{−/−}; Smo:Gli3\textsuperscript{−/−}; Gli2:Gli3\textsuperscript{−/−}) and the talpid2, which lacks an LFP (Litingtung and Chiang, 2000; Wijgerde et al., 2002; Bai et al., 2004; Agarwala et al., 2005). A role for HH signaling in the regulation of cell affinities has been found in the fly wing imaginal disc and abdominal ectoderm (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Lawrence et al., 1999). In each tissue, differential HH signaling creates two compartments which display distinct and inheritable affinities. Thus, cells of a compartment and their lineal relatives cohere with each other and do not intermix with those of the other compartment, although a cell-adhesion molecule involved in these processes that is regulated by HH has not yet been identified.

Recently, HH signaling has also been implicated in the maintenance of orthogonal signaling centers in the vertebrate limb and the MHB of the neural tube (Aoto et al., 2002; Khokha et al., 2003; Blaess et al., 2006). However, whether regulation of boundaries is a general feature of HH action among vertebrates, is not yet known. HH blockade in the midbrain also resulted in a broadened MHB across which cell mixing can occur (Karlstrom et al., 1999; Kiecker and Lumsden, 2005; Zervas et al., 2005). The increased cell mixing noted across the MHB following HH blockade in our experiments therefore suggests a role for HH signaling in limiting such cell-mixing. This is
corroborated in the $Shh^{-/-}$ mouse where MHB cells can be found scattered several cell-diameters away from the MHB (Fogel et al., 2008). In addition to the MHB, we also observed a requirement for HH signaling at the DV boundary (Fig. 3.6). We noted that the DV boundary and the accompanying SERRATE1 expression are also perturbed as a consequence of HH blockade and result in cell mixing. No patterning properties are ascribed to the midbrain DV boundary yet, but SERRATE and NOTCH-DELTA interactions have been implicated in DV patterning in the fly and in the establishment of the AER of the vertebrate limb bud, a signaling center at the DV interface (Irvine and Vogt, 1997). Together, these data suggest that the primary function of the LFP is to properly pattern ventral cell fates and maintain the regional boundaries of the ventral midbrain through an as of yet unidentified mechanism downstream of HH signaling.

**Is LMX1B downstream of FOXA2?**

To our knowledge, this is the first example demonstrating the ability of a single transcription factor to regulate hinge-point formation. FOXA2 and its transcriptional targets therefore become potential candidates in studying epithelial cytoarchitecture and neurulation. Although functional analyses have not been conducted, the gene expression pattern of $LMX1B$ and its presence in ectopic, $FOXA2$-induced hinge-points suggest that it may also play a large role in regulating morphological changes of the neurepithelium (Brown et al., unpublished observations). Early in development, $LMX1B$ is expressed in the isthmus and the median and dorsal lateral hinge-points, each of which are critically important in shaping the midbrain (Fig. 4.6A, B; Yuan and Schoenwolf, 1999). Additionally, LMX1B is important in the specification and
differentiation of dopaminergic neurons, similar to FOXA2 (Sasaki and Hogan, 1994; Smidt et al., 2000; Ferri et al., 2007; Kittappa et al., 2007). Although we are uncertain if LMX1B is a direct target of FOXA2, we have identified several HNF binding sites within the LMX1B gene and its promoter region (Bayly and Agarwala, unpublished observations). Very little is known about LMX1B function outside of dopaminergic neuron production, however, a detailed investigation of LMX1B function is currently underway in our laboratory (Brown et al., unpublished observations).

Why is overexpression of SHH only sufficient to induce an MFP fate in a regionally-restricted manner along the MHB?

It has previously been shown that misexpression of SHH in the midbrain is sufficient to recapitulate the entire pattern of ventral cell fates (Agarwala et al., 2001). However, when we analyzed the effect of SHH overexpression on the MFP, we observed that SHH was only sufficient to induce the MFP in a regionally-restricted manner along the MHB (Fig. 4.3). This observation suggests that a molecule, which is expressed within both the MFP and MHB may be required in addition to SHH to specify an MFP fate. Indeed, several developmental control genes are expressed in each signaling center including BMP7 and LMX1B (Fig. 4.1). Additionally, the extent of MFP induction along the MHB varied between different electroporations, suggesting a possible stage-dependent effect. Most of our electroporations were conducted between H&H 9-11, a time when the MHB is still undergoing refinement (Hidalgo-Sanchez et al., 1999). Earlier in development the MHB is a broad territory, identified by the expression of WNT1 and LMX1B, which encompass the lateral walls of the presumptive midbrain.
and only until H&H 13 is their expression refined caudally (Hollyday et al., 1995; Yuan and Schoenwolf, 1999). Thus a molecule expressed within the MFP and MHB could account for the regionally-restricted manner in which \textit{SHH} can induce an MFP along the MHB (Fig. 4.3). We would then expect that if we coelectroporate \textit{SHH} with an additional gene expressed within the MFP and MHB, we would observe the ectopic induction of MFP throughout ventral midbrain, similar to what is seen with \textit{FOXA2} misexpression (Fig. 4.4). However, as previously mentioned, coexpression of \textit{SHH} with constructs that either constitutively activate BMP signaling or those that prevent BMP activity are not sufficient to induce MFP cell fate specification (data not shown). In contrast, misexpression of \textit{LMX1B} appears sufficient to induce an MFP (Brown et al., unpublished observations). However, it has not yet been tested whether the ability of \textit{SHH} overexpression to induce an MFP along the MHB is dependent upon the presence of \textit{LMX1B}. 
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