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A Novel Role for FOXA2 and SHH in Organizing Midbrain Signaling Centers

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Summary

The floor plate (FP) is a midline signaling center, known to direct ventral cell fates and axon guidance in the neural tube. The recent identification of midbrain FP as a source of dopaminergic neurons has renewed interest in its specification and organization, which remain poorly understood. In this study, we have examined the chick midbrain and spinal FP and show that both can be partitioned into medial (MFP) and lateral (LFP) subdivisions. Although Hedgehog (HH) signaling is necessary and sufficient for LFP specification, it is not sufficient for MFP induction. By contrast, the transcription factor FOXA2 can execute the full midbrain and spinal cord FP program via HH-independent and dependent mechanisms. Interestingly, although HH-independent FOXA2 activity is necessary and sufficient for inducing MFP-specific gene expression (e.g., *LMX1B*, *BMP7*), it cannot confer ventral identity to midline cells without also turning on Sonic hedgehog (SHH).

We also note that the signaling centers of the midbrain, the FP, roof plate (RP) and the midbrain-hindbrain boundary (MHB) are physically contiguous, with each expressing *LMX1B* and *BMP7*. Possibly as a result, *SHH* or *FOXA2* misexpression can transform the MHB into FP and also suppress RP induction. Conversely, HH or FOXA2 knockdown expands the endogenous RP and transforms the MFP into a RP and/or MHB fate. Finally, combined HH blockade and *FOXA2* misexpression in ventral midbrain induces *LMX1B* expression, which triggers the specification of the RP, rather than the MFP. Thus we identify HH-independent and dependent roles for FOXA2 in specifying the FP. In addition, we elucidate for the first time, a novel role for SHH in determining whether a midbrain signaling center will become the FP, MHB or RP.

Keywords

floor plate; midbrain-hindbrain boundary; roof plate; LMX1B; BMP7; signaling centers; spinal cord

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Introduction

During development, the ventral midline of the vertebrate neural tube is occupied by the FP, which secretes SHH and plays a critical role in cell fate specification and axon guidance (His, 1888; Kingsbury, 1920; Placzek and Briscoe, 2005; Strahle et al., 2004). Despite 100 years of study, questions remain unanswered with regard to the embryonic origin, specification and function of the FP across species and axial levels of the neural tube (Placzek and Briscoe, 2005).

The simplest functional organization of the FP to have emerged is its partition into medial (MFP) and lateral (LFP) subdivisions (Odenthal et al., 2000; Schauerte et al., 1998). These divisions are well established in the anamniote and avian spinal FP where the MFP and LFP differ from each other in their embryonic origin, and more controversially, in their dependence upon HH signaling (Charrier et al., 2002; Odenthal et al., 2000; Peyrot et al., 2011; Placzek and Briscoe, 2005; Strahle et al., 2004). HH signaling is required in the fish for specification of the neurectoderm-derived LFP, while the node-derived MFP depends upon Nodal signaling for its induction (Hatta et al., 1991; Odenthal et al., 2000; Schauerte et al., 1998; Strahle et al., 2004). However, these results are complicated by a requirement for HH signaling in the maintenance of MFP at later stages and by the presence of residual FP cells in the *nodal* (*cyclops*) mutant (Odenthal et al., 2000; Ribes et al., 2010).

Gene expression patterns also support a partition of the mouse FP into medial and lateral subdivisions (Odenthal et al., 2000). However, the entire FP disappears following the loss of HH signaling, leaving unanswered the question of whether these subregions differentially require HH signaling for maintenance and/or induction as they do in the fish (Chiang et al., 1996; Fogel et al., 2008; Odenthal et al., 2000).

The chick spinal FP also displays node and neurectoderm-derived MFP and LFP subdivisions (Catala et al., 2000; Charrier et al., 2002). Here, SHH misexpression cannot induce an ectopic MFP, while notochord transplants readily do so via unidentified mechanisms (Catala et al., 2000; Charrier et al., 2002; Gray and Dale, 2010). More recent studies have suggested that early and transient exposure to SHH is capable of inducing a FP in all species, including the bird, fish and mouse (Ribes et al., 2010). But whether SHH is necessary and sufficient for the full FP program has not been addressed.

Even less is known about the specification of anterior (midbrain, rostral hindbrain) FP, which is distinct from the spinal FP in some respects (Patten et al., 2003; Placzek and Briscoe, 2005). For example, in addition to contributions from the node and neurectoderm, the chick anterior FP is also colonized by a unique population of cells emerging from “area a”, a region anterior to Hensen’s node (Patten et al., 2003; Schoenwolf and Sheard, 1990). Interestingly, “area a” explants can be induced to a FP fate in the absence of the notochord by a brief exposure to prechordal plate-derived SHH and Nodal activity (Patten et al., 2003). But whether the anterior FP cell-types are organized into medial and lateral subdivisions and whether they differentially utilize HH and Nodal signaling is not known.

This question has recently acquired significance with the identification of an MFP-like ventral midline region as the predominant source of midbrain dopaminergic (mDA) neurons, which help regulate voluntary movements and the reward-reinforcement circuitry of the brain (Bayly et al., 2007; Blaess et al., 2011; Joksimovic et al., 2009; Ono et al., 2007). Recent work, including ours, suggests that HH signaling may not be necessary for patterning this MFP-like region (Hynes et al., 2000; Kittappa et al., 2007; Lin et al., 2009; Ye et al., 1998). Instead, some studies have reported that the winged helix transcription factor FOXA2 may be a more potent inducer of mDA progenitors than SHH (Kittappa et al., 2007; Lin et al., 2009; Norton et al., 2005; Ribes et al., 2010; Sasaki and Hogan, 1993).

Since SHH and FOXA2 transcriptionally upregulate each other, the differences between their inductive abilities are not well understood (Kittappa et al., 2007; Sasaki et al., 1997). FP studies in the fish suggest that FOXA2 activity can be regulated by, and interacts with, both the SHH and TGFβ/Nodal signaling cascades (Strahle et al., 2004). However, the role of FOXA2 in fish FP specification is complicated by several observations. Although FOXA2 can rescue the MFP in the fish *nodal* (*cyclops*) mutant, *nodal*-dependent induction of FP occurs in the absence of FOXA2 (Norton et al., 2005; Rastegar et al., 2002). Despite its ability to induce the fish MFP, *foxa2* mutants display relatively mild phenotypes consisting mainly of a failure of the FP to fully differentiate (Norton et al., 2005). Although these observations suggest a role for FOXA2 in elaborating, rather than inducing the fish FP, they may also reflect functional redundancy among Foxa2 homologs. This idea is supported by the concurrent downregulation of *Foxa2* and *Foxa3* in the fish, which results in a complete loss of node-derived structures, including the notochord and MFP (Dal-Pra et al., 2011). An identical phenotype is seen in *Foxa2*−/− mutant mice where the absence of the node and notochord also precludes FP specification (Ang and Rossant, 1994; Norton et al., 2005).

Although conditional *Foxa2* or *Foxa1/2* knockouts targeting the ventral midbrain have been created in mice, they are not informative because they disrupt FOXA1/2 activity around E9, when ventral midbrain specification is well underway (Bayly et al., 2007; Blaess et al., 2006; Ferri et al., 2007; Fogel et al., 2008; Lin et al., 2009; Perez-Balaguer et al., 2009). Thus, the precise requirement for SHH and FOXA2 in FP specification remains poorly understood.

In this study, we have approached this problem by comparing the role of SHH and FOXA2 in midbrain and spinal FP specification in the chick, a species in which *FOXA1* or *FOXA3* genes have not been identified. We show that the midbrain FP can be clearly distinguished into MFP and LFP according to multiple criteria. Early manipulations of SHH and FOXA2 exclusively targeted to the neural plate suggest that SHH is not sufficient to induce the MFP in either the midbrain or spinal cord. By contrast, FOXA2 is necessary and sufficient for full MFP and LFP specification and does so via HH-dependent and independent mechanisms.

Notably, we show for the first time that the midbrain signaling centers express a common set of genes, and can take on each others' identities in a SHH-dependent manner. Thus, by regulating the identity of orthogonal signaling centers, MFP signals direct 3-dimensional patterning in the midbrain and hindbrain.

Materials and Methods

Chick embryos

Fertilized Leghorn eggs (Ideal Poultry, Texas) were incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (Hamburger, 1951).

Expression vectors

Embryos were electroporated with EGFP (EFX-EGFP), *Ptc1*$^{\Delta loop2}$ (pCIG-*Ptc1*$^{\Delta loop2}$), *Foxa2* (pMes-Foxa2-IRES-EGFP/EFX-Foxa2), *SHH* (XEX-SHH; pMes-SHH-ires-RFP), dominant negative *Foxa2* (pCAGGS-Fkh^{a2}-IRES-EGFP, pCAGGS-Fkh^{a2}-EnR–IRES-EGFP) or *FOXA2* RNAi (pSilencer-*FOXA2* shRNA) expression vectors (Agarwala and Ragsdale, 2002; Agarwala et al., 2001; Bayly et al., 2007; Briscoe et al., 2001; Jacob et al., 2007). EFX-*Foxa2* was constructed by ligating mouse *Foxa2* cDNA (pBSSK-*mHnf3b*) into EFX3C (Agarwala et al., 2001; Sasaki and Hogan, 1993). pMes-*Foxa2*-IRES-EGFP and pMes-*SHH*-ires-RFP were created by subcloning *Foxa2* or *SHH* cDNAs into the pMes-IRES-EGFP and pMes-IRES-RFP vectors.

FOXA2 shRNA constructs

Four short-hairpin RNA constructs were created by cloning into the pSilencer vector (Ambion). Of these, only one construct (FOXA2-1663) produced effective knockdown of FOXA2 mRNA (Supplementary Fig. S1A, B) and was generated using the following forward: 5′-CTCCTCCTAAAGGCAAAGGTTCAAGAGACCTTTGCCTTTAGGAGGAGTTTTTT-3′ and reverse primers: 5′-AATTAAAAAACTCCTCCTAAAGGCAAAGGTCTCTTGAACCTTTGCCTTTAGGAG GAGGGCC-3′. RNAi electroporations (4 μg/μl) typically produced small effects, but replicated the larger effects of the dnFOXA2 constructs described above (Jacob et al., 2007). These data were therefore pooled.

In ovo electroporation

1–5 μg/μl DNA was electroporated into H&H stages 4–11 midbrains and H&H 8–11 spinal cords according to previous protocols (Agarwala and Ragsdale, 2002; Agarwala et al., 2001; Briscoe et al., 2001; Eom et al., 2011; Momose et al., 1999).

Midbrain explants

Whole embryo (H&H 3–6) or midbrain-hindbrain (H&H 7–9) explants were cultured for 24 hours in 100 μM cyclopamine (Sigma) with 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma) or HBC alone, using established procedures (Agarwala and Ragsdale, 2002; Bayly et al., 2007; Incardona et al., 1998; Norton et al., 2005).

In situ hybridization

Embryos were harvested between E1–E6 and subjected to one and two-color in situ hybridizations according to published protocols (Agarwala and Ragsdale, 2002; Agarwala et al., 2001). The *LMX1A* and *MN-CAD* cDNAs were generated by PCR amplification from E5 midbrains using the forward: 5′-ATGGACAGCGACGATACCTCAC-3′ and reverse: 5′-CCAGACCTACCTCCTGAAACAAGC-3′ primers for *LMX1A* and the forward 5′-CAGATGCTGATGACCCAACCTATG-3′ and reverse 5′-TGATGTGAACGGTGGTGGTGTC3′ primers for *MN-CAD*.

Immunohistochemistry—Embryos were fixed in 4% paraformaldehyde for 15 min-2 hours. 14 μm cross-sections were stained with antibodies against SHH and LMX1B (DHSB; 1:200 each), Alexa-conjugated secondary antibodies were used for fluorescent detection (Afonso and Henrique, 2006). DAPI was used for staining nuclei.

Wholemount p-Smad 1/5/8 immunohistochemistry

Embryos were fixed in 4% PFA for 3 hours and subjected to wholemount p-Smad1/5/8 (1: 2500–5000; Cell Signaling) immunolabeling according to previously established protocols (Eom et al., 2011).

Results

Differential gene expression in medial and lateral FP

We first determined whether the midbrain FP could be divided into medial and lateral divisions by differential gene expression (Odenthal et al., 2000; Placzek and Briscoe, 2005; Strahle et al., 2004). *SHH, FOXA2, LMX1B* and Bone morphogenetic protein 7 (*BMP7*) mRNAs were expressed in the presumptive midbrain FP by H&H 4 and continued to be expressed there until at least E6 (Fig. 1A–I; Supplementary Fig. S2A; Aglyamova and Agarwala, 2007; Echelard et al., 1993; Hamburger, 1951; Lawson et al., 2001; Ruiz i Altaba

et al., 1993; Yuan and Schoenwolf, 1999). By H&H 13, a *SHH*+/*FOXA2*+/*LMX1B*-negative/*BMP7*-negative territory emerged rostrally to surround the *SHH*+/*FOXA2*+/*LMX1B*+/*BMP7*+ FP (Fig. 1E; Aglyamova and Agarwala, 2007). This lateral region expanded caudally toward the MHB, so that it fully encompassed the medial region between late E3–E6 (Fig. 1F, G; Supplementary Fig. S2B, C). Based on gene expression and additional criteria described below, these FP territories were identified as MFP and LFP (Odenthal et al., 2000).

Several other markers, e.g., *LMX1A*, *DISP1*, *MN-CAD* and Chordin, were also expressed in the MFP and distinguished it from the LFP (Fig. 1H, I; Supplementary Fig. S2D; data not shown). Their expression patterns were dynamic and some occupied narrower domains than the *LMX1B* territory, especially at later ages (Fig. 1H, I; Supplementary Fig. S2D; data not shown). A closer examination also showed that the expression of *SHH*, *FOXA2*, *DISP1* and *LMX1B* in MFP cells was mosaic, suggesting that the subarchitecture of the MFP may be more complex than outlined here (Fig. 1H; Supplementary Fig. S2E, E′). However, since our perturbations did not differentially affect the MFP markers identified above, we treated the MFP as one entity in this study.

We were unable to find markers that uniquely and stably identify the LFP for the duration of our experiments. This included *NKX2.*2 expression, which labeled regions outside the FP (Supplementary Fig. S2F). The midbrain LFP was therefore identified by the combined presence of *SHH* and *FOXA2* and the absence of *LMX1A*, *LMX1B*, *DISP1*, *MN-CAD* and *BMP7* (Fig. 1E–J, Supplementary Fig. S2A–D; Table 1; data not shown).

Interestingly, the MFP, RP and MHB all expressed *BMP7* and *LMX1B* and were physically contiguous (Fig. 1K, L; Supplementary Fig. S2B, C). However, *LMX1A*, *DISP1 and MN-CAD* were expressed exclusively in the MFP, while *FGF8* and *GDF7* were expressed exclusively in the MHB and the RP respectively (Fig. 1H, I, M, N; Supplementary Fig. S2D; data not shown). These markers permitted us to unequivocally distinguish the midbrain signaling centers from each other in later experiments (Table 1). These data are summarized in Fig. 1O–Q.

SHH is sufficient for LFP, but not MFP induction in the midbrain

SHH misexpression can induce SHH and FOXA2, both generic markers of the FP, but whether it can induce both midbrain MFP and LFP is not known (Agarwala and Ragsdale, 2002; Agarwala et al., 2001). Unlike controls, *SHH* misexpression (1–5 μg/μl) non-autonomously converted large swaths of midbrain into *SHH*+/*FOXA2*+/*LMX1B*-negative/*DISP1*-negative LFP (Fig. 2A; Agarwala and Ragsdale, 2002; Agarwala et al., 2001). However, *SHH* misexpression could only induce MFP markers (*DISP1, LMX1B*, *BMP7*, *MN-CAD*) along the MHB and nowhere else in the midbrain (Fig. 2A–C; Supplementary Fig. S3A, B, E).

Spinal cord studies have suggested that FP induction is accomplished by early and transient HH signaling prior to somitogenesis (Patten et al., 2003; Ribes et al., 2010). To determine the precise times during which early HH activity might occur in the midbrain, we examined early SHH gene and protein expression. Surprisingly, although *SHH* transcripts were detected as early as H&H 3, no SHH protein or HH receptors (*PTC1*, *PTC2*, *HHIP*) were detected in the axial midline at this time (Fig. 2D–F; Aglyamova and Agarwala, 2007; Lawson et al., 2001; Pearse et al., 2001). Modest levels of SHH protein were first detected in the cytoplasm of most FP and notochord cells at H&H 8^{-}, although some cells remained SHH-negative at this time (Fig. 2G–G″). SHH expression became stronger by H&H 10 and resembled that of the spinal cord, with strong, cytoplasmic expression along the apical surface of FP cells (Fig. 2H, H′; Chamberlain et al., 2008). Robust and transient expression

of *PTC1* and *GLI1* was seen at the ventral midline between H&H 8⁻ to <H&H 10, with more lateral expression seen at later ages (Fig. 2I; Aglyamova and Agarwala, 2007). Together these data defined a narrow window of time during which HH signaling might accomplish midbrain FP induction.

We used a novel early electroporation paradigm to misexpress SHH-ires-RFP between H&H 4–6 to obtain robust *SHH* misexpression by H&H 7, immediately prior to the onset of endogenous SHH protein expression (Brown et al., 2012; Eom et al., 2011). Interestingly, while *SHH* misexpression at this time readily induced ectopic SHH expression and suppressed dorsal expression of LMX1A/B, it failed to induce an LMX1A/B+/SHH+ MFP (Fig. 2J–K′). Thus, early HH activity is not sufficient for midbrain MFP induction, although it is sufficient for LFP induction and suppression of dorsal midbrain fates.

SHH is necessary for midbrain MFP and LFP specification

To determine whether HH signaling was necessary for the specification of the MFP, we misexpressed $Ptc1^{\Delta loop2}$, a dominant negative regulator of HH signaling, between H&H 7–10 when HH signaling is critically required for midbrain cell fate specification (Fig. 2G–I; Bayly et al., 2007). Bilateral misexpression of $Ptc1^{\Delta loop2}$ resulted in a robust disruption of the LFP while leaving *LMX1B* expression in the MFP largely unperturbed (Fig. 3A, B; Bayly et al., 2007).

Despite the lack of evidence of an HH signaling cascade prior to H&H 8⁻, it was possible that low, undetectable levels of early HH activity occurred along the ventral midline and were required for midbrain MFP specification (Ribes et al., 2010). In previous work, we noted that high embryonic lethality was associated with $Ptc1^{\Delta loop2}$ electroporations, and despite robust misexpression throughout the midbrain, only a small number of $Ptc1^{\Delta loop2}$+ cells were found in the ventral midline (Bayly et al., 2007). Possible reasons for this include the differential cell-affinities of HH+ and HH-negative cells, but exclude apoptosis (Bayly et al., 2007; Lawrence, 1997; Rodriguez and Basler, 1997; Wijgerde et al., 2002).

To obtain more ubiquitous HH blockade at early stages, we therefore exposed early whole embryo explants to cyclopamine, a fast-acting inhibitor of HH signaling (Incardona et al., 1998). Although we initially divided the explants into 3 age groups (H&H 3–5, 6–7, 8–9), these data were pooled since all explants displayed similar FP phenotypes in response to cyclopamine. Bath application of 100 μM cyclopamine for 24 hours in H&H 3–9 explants successfully reduced or abolished the expression of HH pathway/target genes, *PTC1*, *DISP1* and *FOXA2* (Fig. 3C–F′; Supplementary Fig. S4A, B; Incardona et al., 1998). However, cyclopamine treatment did not affect *LMX1B* expression at the ventral midline (n=16/18; Fig. 3C–D′; Pearse et al., 2001). Together, the gain and loss of function experiments reported above suggest that regardless of the age of manipulation, HH activity was necessary, but not sufficient for the full execution of the MFP program.

Since the RP, FP and MHB all express *LMX1B*, we next asked whether the *LMX1B* expressing ventral midline retained FP identity following HH blockade (Fig. 1K, L; data not shown; Chizhikov and Millen, 2004). To our surprise, the cyclopamine-treated explants displayed ectopic *GDF7* expression in ventral midline cells in which *FOXA2* expression had been abolished, suggesting that some FP cells acquired ectopic RP identity in the absence of HH signaling (Fig. 3E–G′). $Ptc1^{\Delta loop2}$ electroporations at H&H 7–11 provided additional evidence for the conversion of some FP cells into *GDF7*+ RP cells (Fig. 3H, I; arrowheads in 3I). With the exception of *GDF7*, exclusive RP markers that were not also expressed in other midbrain signaling centers were not available (Fig. 1K–Q; Table 1). However, the ectopic ventral midline expression of *WNT1* (which is expressed in the RP and MHB, but not the avian FP; Fig. 1M; Fig. 7D) and *WNT1*+/*GDF7*+ cells in the ventral midline

following *Ptc1*$^{\Delta loop2}$ electroporations further confirmed the transformation of FP cells into RP identity (Supplementary Fig. S5A, B; Fig. 6H).

To determine whether the ectopic ventral midline expression of *GDF7* represented a specific conversion of the MFP to the RP or a generic ventral to dorsal conversion in the absence of HH activity, we examined *PAX7* expression, which is absent in the ventral midbrain as well as RP, but otherwise present throughout the dorsal midbrain (Fig. 3J). Interestingly, cyclopamine treatment resulted in ectopic *PAX7* expression throughout ventral midbrain except for the *LMX1B*+ region along the ventral midline (Fig. 3K, L). The presence of *GDF7*+/*WNT1*+/*LMX1B*+/*PAX7*-negative cells in the MFP demonstrated that in the absence of HH signaling, the ventral midline of the midbrain was specified as RP rather than as MFP. Taken together, these results show that HH signaling is necessary, but not sufficient to execute the full midbrain MFP program (Charrier et al., 2002; Peyrot et al., 2011; Ribes et al., 2010).

FOXA2 induces the full midbrain FP program via HH-dependent and independent mechanisms

FOXA2 is necessary and sufficient to induce MFP and LFP in ventral midbrain —Unlike SHH, FOXA2 mRNA and protein expression are seen at the axial midline of the embryo at H&H 2, prior to the activation HH signaling (Fig. 4A, B; Fig. 2E, G; Ruiz i Altaba et al., 1993). Given our limited understanding of FOXA2 function in regionalizing the amniote FP, we asked whether FOXA2 might induce midbrain MFP (Ang and Rossant, 1994; Norton et al., 2005; Ribes et al., 2010; Sasaki and Hogan, 1993; Sasaki and Hogan, 1994; Strahle et al., 2004). Interestingly FOXA2 electroporations early (H&H 4–6) or late (H&H 7–11) non-autonomously converted ventral midbrain into FP (Fig. 4C; 4D, inset 4D; Supplementary Fig. S6A, B). Unlike *SHH* however, *FOXA2* was sufficient to induce both LFP and MFP markers throughout the midbrain and not just along the MHB (Fig. 4C–F; Fig. 2A–C; Supplementary Fig. S3A–F). Conversely, dominant negative FOXA2 (dnFOXA2) and FOXA2 RNAi electroporations between H&H 4–11 prevented the induction of both the MFP and the LFP (Fig. 4G, H; control for 4H is shown in Fig. 4K; Supplementary Fig. S6A, C).

FOXA2 cannot ventralize the FP without turning on SHH—We next determined the extent to which the *FOXA2*-mediated induction of MFP depended upon HH signaling. Joint electroporations of *FOXA2* and *Ptc1*$^{\Delta loop2}$ mimicked *FOXA2* misexpression by inducing *LMX1B* expression throughout ventral midbrain, demonstrating that *LMX1B* induction by FOXA2 is HH-independent (Fig. 4I, J). However, in contrast to *FOXA2* misexpression alone, ectopic *LMX1B*+ puncta induced by *FOXA2*/*Ptc1*$^{\Delta loop2}$ co-electroporations also expressed *GDF7*, an exclusive marker of midbrain RP (Fig. 4I, J, arrowheads). Thus, following joint *FOXA2* misexpression and HH blockade, cells ectopically expressing *LMX1B* adopt a RP, rather than a FP identity. We conclude that FOXA2 is necessary for midbrain MFP and LFP induction, and is necessary and sufficient to induce MFP-specific genes in the absence of SHH. By contrast, HH activity is required for inducing HH-pathway genes (*DISP1*, *PTC1*, *FOXA2*), without which the ventral midline cannot acquire a specific MFP identity (Fig. 3C–I). Thus the HH pathway is required for LFP specification and the ventralization of the MFP, while HH-dependent and independent activities of FOXA2 are required for the execution of the full midbrain FP program.

FOXA2 is necessary to restrict MFP induction to the ventral midline of the midbrain—A HH-dependent lateral expansion of *netrin1* and *nkx2.2a* is seen in fish *foxa2* (*monorail*) mutants, suggesting a role for FOXA2 in limiting HH activity laterally and preventing the lateral expansion of FP (Norton et al., 2005). In agreement with these

observations, dnFOXA2 and FOXA2 RNAi-electroporated midbrains demonstrated a non-autonomous induction of MFP in lateral midbrain (n=8; arrow, Fig. 4H, arrowhead, Fig. 4L). Interestingly, such non-autonomous induction of the MFP was not seen in embryos electroporated with *Ptc1*$^{\Delta loop2}$, suggesting that it was likely to be HH-dependent (Fig. 3B). Thus, in addition to its role in inducing the MFP and LFP, FOXA2 also plays a role in restricting the MFP domain to the ventral midline of the midbrain, as suggested by mouse *Foxa2* enhancer analyses and fish *foxa2* mutants (Norton et al., 2005; Sasaki et al., 1997).

FOXA2, but not SHH, is sufficient for specifying the full spinal FP program

In the chick spinal cord, a notochord transplant can induce a complete FP, while ectopic SHH can only induce the LFP (Charrier et al., 2002). Since *FOXA2* is expressed in the spinal FP and notochord, we next asked whether SHH and FOXA2 might play different roles in spinal FP specification as they do in the midbrain. Like the midbrain, we noted the presence of an *LMX1B*+/*SHH*+ MFP and *LMX1B*-negative/*SHH*+ LFP in the spinal cord (Fig. 5A). Unilateral electroporation of *SHH* in the H&H 10 rostral spinal cord suppressed dorsal fates (e.g. dI5 interneurons, arrowhead; Fig. 5A, A′) and readily induced ectopic *LMX1B*-negative/*SHH*+ LFP (Fig. 5A, A′). However, it did not induce an *LMX1B+/SHH+* MFP (n= 10/10; Fig. 5A, A′). By contrast, *FOXA2* misexpression induced both ectopic MFP and LFP, in addition to recapitulating the characteristic FP morphology (Fig. 5B, B′). Taken together, our observations suggest that FOXA2, but not SHH, is sufficient to execute the entire FP program in the midbrain and spinal cord.

SHH and FOXA2 regulate the specification of all midbrain signaling centers

The midbrain-hindbrain boundary—In Fig. 2 and 4, we demonstrated that ectopic SHH and FOXA2 can induce an MFP along the MHB, although how this occurs was not explored (Figs. 2A–C; 4D). *FOXA2* or *SHH*-mediated induction of MFP markers along the MHB resulted in a concurrent suppression of the MHB markers, *FGF8* and *WNT1* (Fig. 6A, B; Supplementary Fig. S5C). To determine whether a functional MFP formed along the MHB, we asked whether the ectopic MFP provided axon guidance cues, a known function of the FP (Charron et al., 2003). Immuno-labeling by phosphorylated-SMAD 1/5/8 (a readout of canonical BMP signaling) allows for the identification of multiple axon trajectories in the midbrain, either attracted or repelled by FP signals (Fig. 6C; Fedtsova et al., 2008). Interestingly, *FOXA2*-mediated induction of the MFP along the MHB resulted in a complete recapitulation of axon trajectories seen at the MFP, while repressing those seen at the MHB (Fig. 6C, right side). Thus, FOXA2 and SHH suppress the MHB program and convert it into a functional MFP, which can redirect axon trajectories.

By contrast, dnFOXA2 resulted in a massive induction of *WNT1* and *FGF8*, converting the FP into MHB and the midbrain into hindbrain (black arrowheads, Fig. 6D, E and red arrowhead, Fig. 6D; see also Fig. 6G; Agarwala and Ragsdale, 2002; Liu et al., 1999). We noted that this MHB induction was non-autonomous, but required that the dnFOXA2 electroporations were targeted to the ventral midline (Fig. 6E, F). By contrast, no induction of *FGF8* occurred when dnFOXA2 was misexpressed outside the FOXA2 domain (Fig. 6F). We conclude that the non-autonomous induction of *WNT1* and *FGF8* throughout the midbrain was an indirect consequence of converting the MFP into the MHB and of expanding the endogenous MHB.

FGF8 misexpression is known to convert the midbrain into hindbrain by inducing *GBX2* (Liu et al., 1999). Indeed, dnFOXA2 cell-autonomously upregulated *GBX2* and suppressed *OTX2*, converting midbrain into hindbrain, while non-autonomously upregulating *OTX2* (Fig. 6G; data not shown). HH blockade by *PTC1*$^{\Delta loop2}$ replicated the effects of dnFOXA2 and also converted the MFP into MHB (Fig. 6H; Bayly et al., 2007). Together, these data

suggest that FOXA2 and HH signaling are involved in determining whether a midbrain signaling center will take on MHB or MFP fates.

The roof plate—We showed above that HH mediated-ventralization of the midbrain determined whether the *LMX1B+* ventral midline expressed MFP or RP fates (Fig. 3E–I; Fogel et al., 2008). We explored the inter-convertibility of MFP and RP further with SHH gain and loss of function experiments. Ventral *SHH* misexpression suppressed the specification of a *GDF7+/WNT1+/LMX1B+* RP (Fig. 7A–E). Interestingly, very small ventral electroporations of SHH were sufficient to non-autonomously suppress RP specification (Fig. 7D, E, inset E). Conversely, ventral electroporations of $Ptc1^{\Delta loop2}$resulted in an expansion and ectopic induction of the midbrain RP, a phenotype similar to the RP expansion noted in *Shh−/−* mice (Fig. 7F; Fogel et al., 2008). Ventral FOXA2 blockade mimicked the effects of HH blockade by expanding the endogenous midbrain RP and by ectopically inducing RP in dorsal midbrain (Supplementary Fig. S5D).

Surprisingly, suppression of HH activity along the MHB also resulted in a conversion of the MHB into *GDF7+* RP cells (Fig. 7G–I, arrow in 7I). Together, these results suggest that SHH plays a role in repressing the RP program dorsally and along the MHB. In its absence the RP expands and the MHB and the MFP take on RP identity. These results suggest that HH is required for the specification and/or maintenance of all midbrain signaling centers, including the FP, RP and the MHB.

Discussion

Our results suggest that SHH is both necessary and sufficient for LFP induction, but it is only necessary, and not sufficient for inducing the MFP program (Odenthal et al., 2000; Strahle et al., 2004). By contrast, FOXA2 is necessary and sufficient to specify the entire FP and does so in a HH-dependent and independent manner (Fig. 8). We have also identified a novel function for HH signaling in determining the identity of midbrain signaling centers, with critical consequences for 3-dimensional midbrain patterning and for midbrain and hindbrain identity (Fig. 8).

The role of FOXA2 and SHH in FP specification

The role of SHH in MFP specification has been difficult to ascertain, with evidence ranging from an absolute requirement for HH signaling in the mouse, to a role in MFP maintenance in the fish (Blaess et al., 2006; Fedtsova et al., 2008; Fogel et al., 2008; Odenthal et al., 2000; Perez-Balaguer et al., 2009). In this study, we examined the precise requirement for HH signaling in midbrain MFP specification and show that it is required for ventralizing the MFP by turning on HH pathway and target genes (e.g., *SHH, FOXA2*, *DISP1*). However, it is insufficient for executing the full MFP program because it cannot turn on MFP-specific genes such as *DISP1*, *MN-CAD*, *LMX1B*, *LMX1A* and *BMP7*. We also show that HH blockade experiments between H&H 3–21 result in a loss of LFP, but do not eliminate all MFP markers (Bayly et al., 2007). Thus our data suggest that while HH signaling is necessary for MFP and LFP specification, it is only sufficient for LFP, but not MFP specification.

In this and previous work, we have also addressed the issue of the timing and duration of HH requirement (Bayly et al., 2007). Some studies have suggested that FP induction is accomplished at presomitic stages by a brief, but intense pulse of SHH (Patten et al., 2003; Ribes et al., 2010). Interestingly, we and others have shown that although SHH transcripts are present at the axial midline by H&H 3, the HH signal transduction machinery (*PTC1*, *PTC2*, *HHIP*, *GLI1*) is not present until H&H 5 (Aglyamova and Agarwala, 2007; Pearse et al., 2001). Surprisingly, the onset of SHH protein expression in the axial midline occurs

later, at H&H 8^{-}, when strong *GLI1* and *PTC1*, transcriptional targets and readouts of HH signaling, are also transiently seen at the FP (H&H 8 to <H&H 10; Aglyamova and Agarwala, 2007; Pearse et al., 2001).

This post-somitic expression of SHH proteins in the chick is in contrast to the presomitic SHH expression reported in the mouse, and suggests that HH-mediated FP specification in the midbrain may occur at different time points in these species (Blaess et al., 2006; Fogel et al., 2008; Marti et al., 1995; Placzek and Briscoe, 2005; Ribes et al., 2010). Furthermore, neither the midbrain FP nor the subjacent notochord expresses the high levels of SHH seen at spinal cord levels (Ribes et al., 2010). However, it is still possible that midbrain FP patterning requires early and low/undetectable levels of the HH signaling emanating from the node or notochord (Jeong and McMahon, 2005; Placzek and Briscoe, 2005). Even so, HH signaling is still only necessary for the induction of HH-target/pathway genes at the ventral midline and not for the induction or blockade of MFP-specific markers such as *LMX1B* and *BMP7*. Thus the principal role of HH signaling in MFP patterning appears to be its ability to ventralize the FP. The precise molecular mechanisms underlying midbrain ventralization have not been extensively studied, but are likely to include the induction of HH pathway genes (*DISP1*, *PTC1*) and the exclusion of dorsal markers (*PAX7*) from the ventral neural tube (Bayly et al., 2007; Ericson et al., 1997; Fogel et al., 2008).

HH-independent mechanisms in the ventral midbrain patterning have also been noted in the *Shh*$^{-/-}$ and *Smo*$^{-/-}$ mice (Fogel et al., 2008). These mutants do not express *Gli1* and *Ptc1* in the FP and the entire ventral midbrain is dorsalized (*Pax7*+) by E9 (Blaess et al., 2006; Fogel et al., 2008). Although these results could be interpreted to signify an absolute requirement for HH signaling in the midbrain FP, a *Pax7*-negative ventral midbrain region is initially specified in the mouse, but it cannot be maintained beyond E9 without HH signaling (Blaess et al., 2006; Chiang et al., 1996; Fedtsova et al., 2008; Fogel et al., 2008). Taken together, these data may reconcile the apparent differences in chick and mouse midbrain FP specification by suggesting that the FP can be initiated in the absence of HH signaling, but requires HH signaling for its ventralization in both amniotes. A full comparison between the chick and mouse FP however would require knowing whether MFP-specific gene induction also occurs in the *Shh*$^{-/-}$ and *Smo*$^{-/-}$ mice, but these data are currently not available.

FOXA2 is both necessary and sufficient for turning on SHH in the midbrain, but accomplishes the ventral midline induction of *LMX1B* and *BMP7* in a HH-independent manner. Its ability to induce *LMX1B*, a critical regulator of mDA neuron induction, may account for its greater potency in inducing mDA neurons (Andersson et al., 2006; Lin et al., 2009; Nakatani et al., 2010; Smidt et al., 2000; Yan et al., 2011). Whether FOXA2 interacts with the Nodal pathway in the chick midbrain is currently under study in our laboratory. Previous studies suggest that joint misexpression of Nodal and SHH is sufficient to induce “area a” cells to a FP fate, but whether FP subregions differentially depend upon Nodal and/or SHH signaling is not known (Patten et al., 2003). Our preliminary data suggest that multiple TGFβ ligands and their essential effectors (phosphorylated SMAD 2, 3) are active throughout the midbrain and are essential early regulators of *FOXA2* expression in the FP (Amarnath et al., unpublished observations). Given their widespread distribution, any specific action of Nodal/TGFβ signaling within the FP is likely to be mediated via regulation of FOXA2 at the axial midline.

Since SHH can induce FOXA2, why is SHH not as potent as FOXA2 in MFP induction? One reason for this is that SHH protein is not expressed in the axial midline at early stages when FOXA2 protein expression is clearly detected (this study; Ruiz i Altaba et al., 1993). Since onset of *LMX1B* and *BMP7* mRNA expression at the ventral midline occurs prior to SHH protein expression and FOXA2 can induce these and other MFP markers in a HH-

independent manner, FOXA2 is likely to initiate the MFP program in the midbrain and not SHH (Amarnath et al., unpublished data; Yuan and Schoenwolf, 1999). However, once expressed, SHH must rapidly take control of *FOXA2* expression since cyclopamine-treated explants collected at H&H 9, one stage (~90 min) after the onset of SHH protein expression, display severely reduced FOXA2 (Fig. 3F). This short time however is not sufficient to turn down *LMX1B* expression in such explants, despite the critical role of FOXA2 in its induction (Fig. 4A–H; Supplementary Fig. S6A–C).

The role of SHH and FOXA2 in regulating midbrain's signaling centers

Signaling centers that pattern a given tissue are known to cross-regulate each other. Such crosstalk is a key to establishing a 3-dimensionally correct tissue pattern and has been noted between the signaling centers of the limb (Benazet and Zeller, 2009; Duboc and Logan, 2011). Evidence has also recently accumulated that the modulation of one of midbrain's signaling centers affects the patterning or maintenance of other signaling centers (Aoto et al., 2002; Basson et al., 2008; Blaess et al., 2006; Blaess et al., 2008; Fogel et al., 2008).

A clear role for SHH in the maintenance of the MHB and suppression of the RP program has been established in the chick and/or mouse (Bayly et al., 2007; Blaess et al., 2006; Fogel et al., 2008). Ventral blockade of SHH and FOXA2 cause an expansion and ectopic induction of RP in dorsal midbrain in both chick and mouse, while their overexpression suppresses RP induction (data not shown; Fogel et al., 2008). Although this could be interpreted as a generic role for SHH in ventralization, our previous data demonstrate specific up and downregulation of dorsal midbrain genes by SHH (Fogel et al., 2008). Whether SHH regulates RP specification directly or indirectly is not known, although a transient, early wave of SHH activity floods and then recedes from the dorsal neural tube, and HH effectors, *GLI1-GLI3* are all expressed dorsally (Aglyamova and Agarwala, 2007; Chamberlain et al., 2008). Finally, multiple HH-binding proteins and pathway members, which could potentially transduce HH signals (e.g., Megalin, *BOC*), are also expressed in dorsal midbrain (Aglyamova and Agarwala, 2007).

In this study, we show that ventral midline cells can become MFP or RP cells, depending on whether HH signaling is present or absent. Why only a handful of MFP cells take on the RP phenotype is not understood. Given the mosaic nature of FP gene expression, one possibility is that only a subset of *LMX1B*+ MFP cells are competent to turn on RP fates in the absence of HH signaling. Alternatively, we and others have noted the scarcity of HH-negative cells at the ventral midline following attempts to block HH signaling in this region (Bayly et al., 2007; Briscoe et al., 2001; Wijgerde et al., 2002). This may be due to the differential cell-affinity based segregation of HH-negative and HH+ cells as noted in the fly (Rodriguez and Basler, 1997). In this scenario, only a few HH-negative cells would remain at the ventral midline, although all of these would turn on RP fates in the absence of HH signaling.

The midbrain-hindbrain boundary—Previous studies suggest that although SHH is not required for MHB induction, it is required for maintaining its spatial coherence and signaling properties (Aoto et al., 2002; Bayly et al., 2007; Blaess et al., 2006; Blaess et al., 2008). In the mouse, the absence of SHH results in a progressive loss of FGF8 and a near complete loss of *WNT1* in the MHB by E12.5 (Blaess et al., 2006; Fogel et al., 2008). Interestingly, *GLI1-GLI3 and Megalin* are all expressed within the chick MHB at various time points, and MHB regulation by GLI3 has been clearly demonstrated (Aglyamova and Agarwala, 2007; Aoto et al., 2002; Blaess et al., 2008). Here we extend these observations to show that overexpression of SHH blocks MHB induction by converting the MHB into a MFP fate. Together these results show that the MHB is sensitive to both increased and

decreased levels of HH signaling, being converted to MFP in the presence of excess SHH and losing its signaling properties when SHH is downregulated.

More surprisingly, the absence of HH signaling can also convert the MFP into the MHB. In such experiments, the MHB can also be converted into a *GDF7+/WNT1+* RP. Fate mapping and lineage analyses suggest a common set of precursors for the MHB and the RP, which might explain their inter-convertibility in our experiments (Alexandre and Wassef, 2003; Zervas et al., 2005). To our knowledge, no common RP/MFP or MFP/MHB precursors have been described. However, as we demonstrate in Fig. 1K, L, all 3 signaling centers are physically contiguous and cell exchange among them is therefore feasible. We also note that all midbrain signaling centers express a common set of developmental control genes (*BMP7*, *LMX1B*). Although the significance of *BMP7* expression in all 3 signaling centers is not understood, LMX1B is clearly involved in FP, MHB and RP specification (Adams et al., 2000; Chizhikov and Millen, 2004; Matsunaga et al., 2002; Millen et al., 2004; O'Hara et al., 2005; Yan et al., 2011). Thus, LMX1B may be a key regulator of midbrain signaling center induction and may specify the MFP, MHB or RP depending upon the specific cellular context. If so, a key component of that cellular context would be the presence or absence of SHH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

bi	bilaterally electroporated
CYC	cyclopamine
DI	diencephalon
E	embryonic day
EP	electroporated
FP	floor plate
HH	hedgehog
H&H	Hamburger & Hamilton
HB	hindbrain
hn	Henson's Node
LFP	lateral floor plate
MB	midbrain
me	mesendoderm
MFP	medial floor plate

MHB	midbrain-hindbrain boundary
ne	neurectoderm
RP	roof plate
TEC	tectum

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Highlights

- We identify medial and lateral subdivisions in avian midbrain and spinal floor plate
- These subdivisions differentially require FOXA2 and SHH for their induction
- Only FOXA2 induces the full floor plate via SHH independent and dependent mechanisms
- The medial region shares gene expression with other midbrain signaling centers
- Midbrain signaling centers are interconvertible and depend on SHH for their identity >

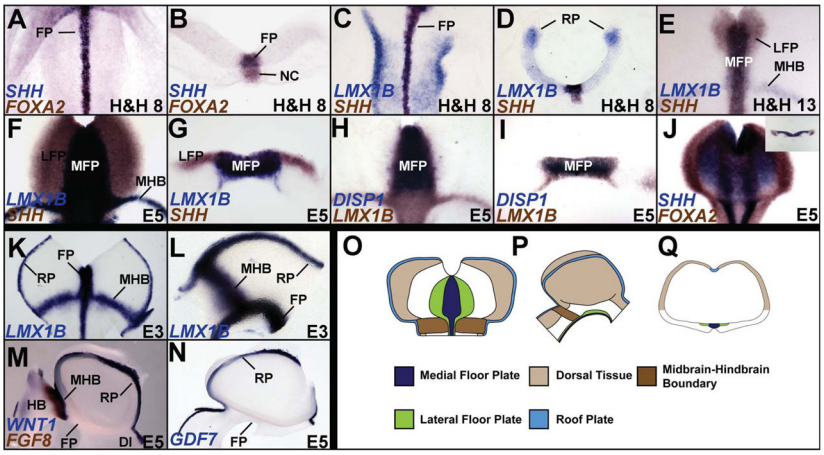

Figure 1.

A–J. Gene expression patterns distinguish the midbrain MFP and LFP. *SHH*, *FOXA2*, *LMX1B* and *DISP1* expression in the FP. A: Whole embryo, top-down view; B, D, G, I, inset J: cross-sections; C, E, F, H, J: flattened wholemounts (open book preparation, rostral to the top and ventricular surface facing the viewer). **K–N. Overlapping and specific gene expression patterns in midbrain signaling centers.** Flattened wholemount (K) and sagittal (L–N) views displaying physically contiguous *LMX1B* expression in the MFP, RP and MHB (K, L), the exclusive expression of *FGF8* in the MHB (M) and of *GDF7* in RP (N). Note that *WNT1* is expressed in the RP and MHB, but not FP. **(O–Q)** Cartoons displaying the 3-dimensional spatial relationships among the midbrain signaling centers. O: flattened wholemount; P: sagittal view, rostral to the right; Q: cross-section.

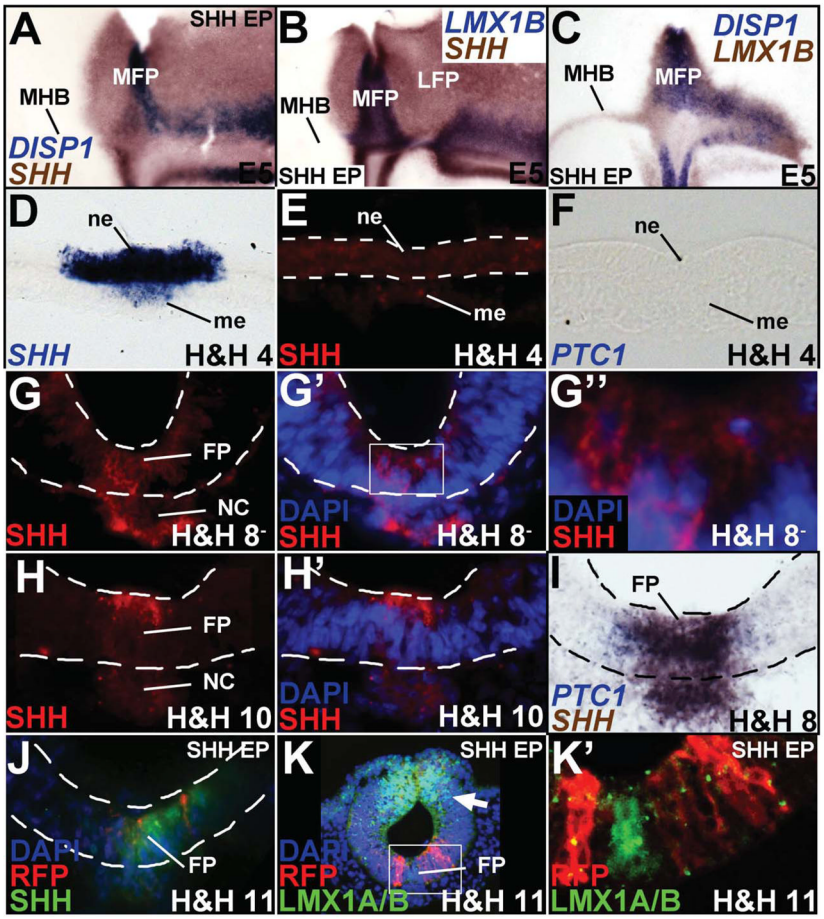

Figure 2. SHH misexpression is sufficient for LFP, but not MFP induction

(A–C, flattened wholemounts, oriented as in Fig. 1C; D–K′, cross-sections). **(A–C)** Unilateral *SHH* misexpression is sufficient to convert the entire right half of the midbrain into *SHH*+ LFP (A, B), but can only specify an *LMX1B*+/*DISP1*+/*SHH*+ MFP along the MHB (A–C). **(D–F)** *SHH* mRNA (D) but no SHH protein (E) or *PTC1* transcripts (F) at H&H 4. Sections in D–F are drawn from presumptive neural plate anterior to Hensen's node. **(G–H′)** SHH protein (red) at H&H 8⁻ (G–G″) and H&H 10 (H–H′). G′ and G″ show the micrograph in G with DAPI staining. The boxed area in G′ is magnified in G″ to show a SHH+ cell (arrowhead) and a SHH-negative cell (arrow). H′ shows the image in H with DAPI staining. **(I)** *SHH* and *PTC1* mRNA at H&H 8. **(J, K′)** H&H 4–6 *SHH*-ires-*RFP* electroporation (red) is sufficient for ectopic, non-autonomous SHH (J, green) induction and dorsal LMX1A/B suppression (arrow, K), but it is not sufficient for LMX1A/B (green) induction ventrally (K, K′). Boxed area in K is magnified in K′ to show the absence of LMX1A/B induction in or around SHH -ires-RFP+ cells. Please note that the sections shown in G–K′ are drawn from the rostrocaudal midpoint of the midbrain.

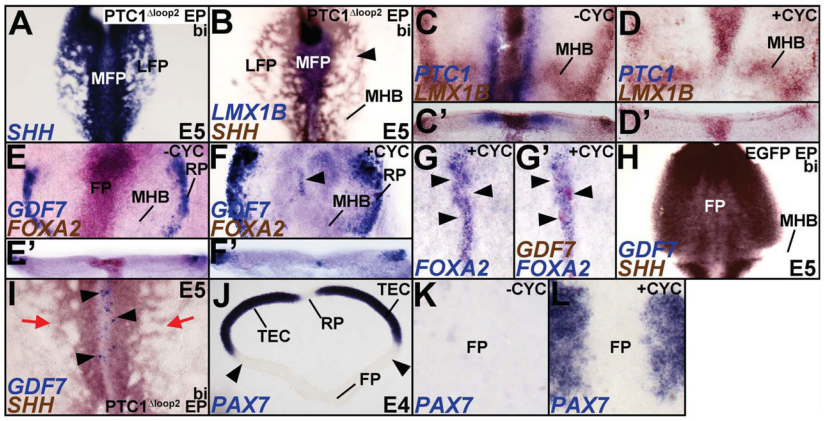

Figure 3. SHH is necessary for MFP induction

(A–B) Broad, bilateral misexpression of *PTC1?$^{\Delta loop2}$* disrupts the *SHH+/LMX1B*-negative LFP (B, arrowhead), but does not affect the *SHH+/LMX1B+* MFP. Control for 3B can be seen in Figure 4E. **(C, D)** H&H 3 explants treated with either vehicle (−CYC) or cyclopamine (+CYC) for 24 hours demonstrate that HH blockade suppresses *PTC1* expression in LFP, but does not affect *LMX1B* expression in the MFP. **(C′, D′)** Cross-sections of the wholemounts shown in C and D. **(E, E′)** Control explants in flattened wholemount (E) and cross-sectional (E′) view displaying *GDF7* (RP) and *FOXA2* (FP) expression. **(F, F′)** Cyclopamine treated explants displaying the loss of *FOXA2* and the ectopic induction of *GDF7* at the ventral midline (arrowhead). Note that compared to E, E′, the roof plate (RP) expression of GDF7 is expanded in F, F′. **(G, G′)** Cyclopamine treated explants with mild HH blockade showing ectopic expression of *GDF7* (arrowheads) in the ventral midline whereever *FOXA2* expression is abolished. Note that G and G′ show the same explant before and after *GDF7* staining. Control for G, G′ is shown in Fig. E. **(H)** Control brain demonstrating that *GDF7* is not expressed in the *SHH+* FP or MHB. See also, 1N; 7G, H for controls. **(I)** Bilateral HH blockade results in a disruption of the LFP (red arrows) and a conversion of the MFP into *GDF7+* RP (arrowheads). **(J)** Midbrain cross-section demonstrating that *PAX7* expression is absent from the RP and ventral midbrain, but present throughout dorsal midbrain (TEC). **(K, L)** Control (K) and cyclopamine treated explants (L) shown in wholemount view demonstrating that the ventral expansion of *PAX7* into ventral midbrain (K) excludes the ventral midline. The *PAX7*-negative region between the arrowheads in J was flattened in K, L.

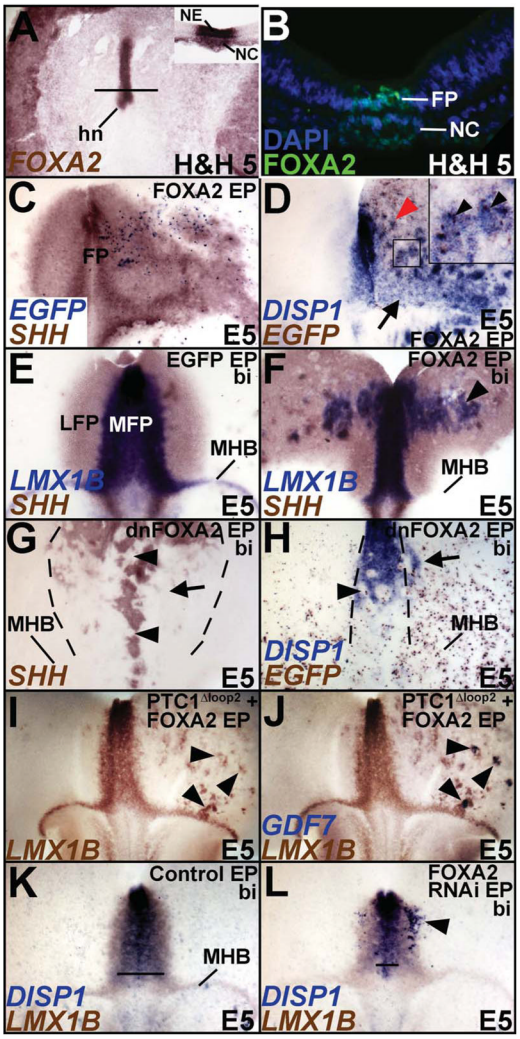

Figure 4. Foxa2 is necessary and sufficient for midbrain FP specification

(A, B) *FOXA2* mRNA (A, inset A) and protein (B) are expressed in the axial midline of the embryo by H&H 5, prior to HH signaling. A: Whole embryo, top down view. Inset, A: Section taken from this embryo at the level indicated by the black line. **(C)** Unilateral misexpression of *FOXA2-ires-EGFP* (right) is sufficient to non-autonomously upregulate *SHH*. **(D)** Unilateral misexpression of FOXA2-ires-EGFP (right) results in *DISP1* upregulation both along (arrow) and away (red arrowhead) from the MHB. Boxed region in D is magnified in the inset to demonstrate the non-autonomous induction of *DISP1* (blue) by FOXA2-ires-EGFP (brown) misexpressing cells. **(E, F)**. EGFP (E) and bilateral *FOXA2* misexpression (F) demonstrating that ectopic *FOXA2* can induce an *LMX1B*+/*SHH*+ MFP away from the MHB (arrowhead, F). **(G, H)** Bilateral *dnFOXA2* electroporation (identified by EGFP detection in H) is sufficient to block LFP (arrow, G) and MFP (arrowheads, G, H) induction. Arrow in H points to the non-cell autonomous expansion of *DISP1* following *FOXA2* knockdown. **(I, J)** Unilateral $PTC1^{\Delta loop2}$ and *FOXA2* co-electroporation demonstrates that *FOXA2*-mediated induction of *LMX1B* is HH-independent. Note that the cells ectopically inducing *LMX1B* also express *GDF7* and adopt a RP fate (arrowheads) in the absence of HH. The same wholemount is displayed in G and H before and after *GDF7* detection. **(K, L)** Compared to controls (K), FOXA2 RNAi results in the suppression of

MFP (compare the lines in K and L, marking the mediolateral extent of *DISP1*) as well as the ectopic induction of MFP in lateral midbrain (arrowhead, L).

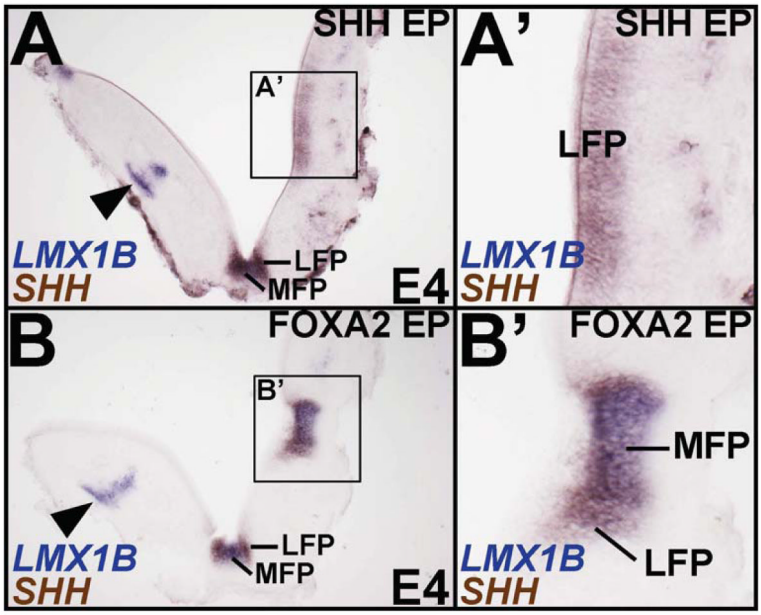

Figure 5. FOXA2 is sufficient for MFP and LFP induction in the spinal cord

Boxed areas in A and B are magnified in A′ and B′, respectively. **(A, A′)** Unilateral *SHH* misexpression (right side) is sufficient to induce a *SHH*+ LFP (boxed area, A; A′), but fails to induce a *SHH*+/*LMX1B*+ MFP. **(B, B′)** *FOXA2* misexpression (right side) in the spinal cord is sufficient to induce both the MFP and LFP (boxed area, B; B′), accompanied by a FP-like morphology. Note that unlike the control side (arrowheads, A, B) *FOXA2* and *SHH* both suppress *LMX1gB*+ dorsal dI5 interneurons.

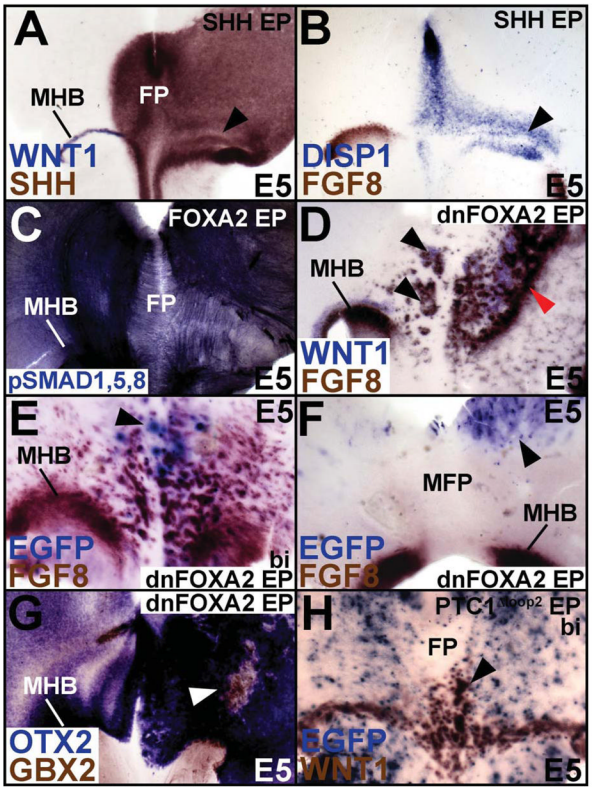

Figure 6. SHH and FOXA2 regulate the signaling centers of the midbrain

(A, B) Unilateral *SHH* misexpression (right side) induces the MFP (*DISP1*+/*SHH*+) while concurrently suppressing MHB (*FGF8*+, *WNT1*+) fates (arrowheads, A, B). **(C)** *FOXA2* misexpression converts the MHB (right side) into an ectopic MFP, which recapitulates the endogenous MFP pattern of axons, identified by phosphorylated-SMAD 1/5/8. **(D)** dnFOXA2 misexpression can convert the FP into MHB (arrowheads). Note also that the MHB is expanded and shifted rostrally (red arrowhead) on the right, most likely due to ectopic hindbrain induction caudal to it (See also Fig. 6G). **(E, F)** Ectopic MHB can only be induced by dnFOXA2 misexpression within the *FOXA2*+ FP (arrowhead, E) and not outside it (arrowhead, F). **(G)** dnFOXA2 suppresses *OTX2+* midbrain fate and upregulates *GBX2*+ hindbrain fates (arrowhead). **(H)** Bilateral HH blockade also results in the conversion of caudal MFP into MHB (arrowhead).

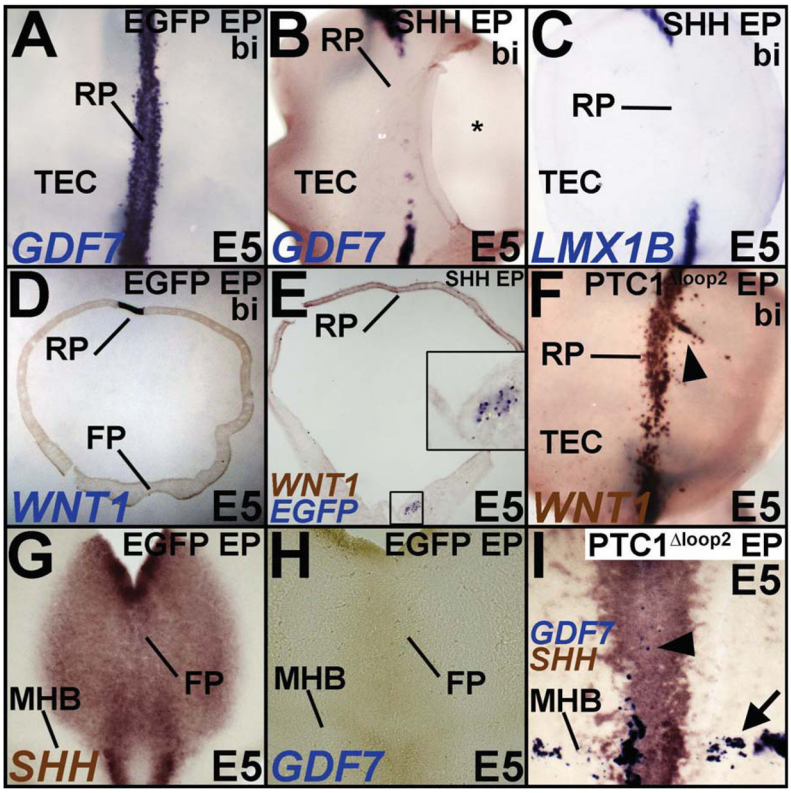

Figure 7.

(A–E) Top down (A–C) and cross-sectional views (D, E) demonstrating that compared to EGFP-electroporated controls (A, D), RP induction is blocked by ventral electroporations of SHH. Inset in E shows magnified view of the boxed region and demonstrates the location of SHH misexpression by EGFP transgene expression. * in B marks a hole cut in dorsal midbrain for irrigation. **(F)** Ventral *PTC1*$^{\Delta loop2}$ misexpression results in ectopic RP induction (arrowhead). **(G–I)** Midbrain flatmounts showing that unlike controls (G, H), *PTC1*$^{\Delta loop2}$ misexpression results in reduced *SHH* expression accompanied by ectopic *GDF7* expression in both the ventral midline (arrowhead) and the MHB (arrow).

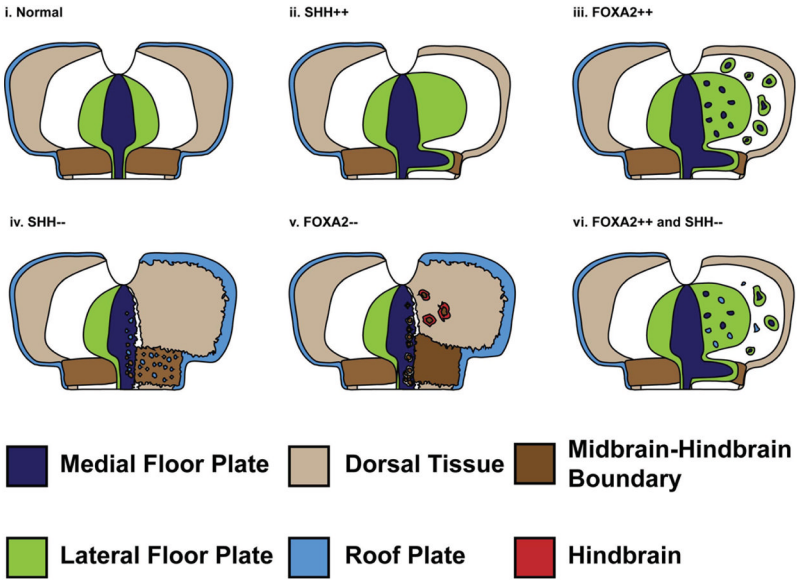

Figure 8. Cartoon summarizing the role of SHH and FOXA2 in patterning the MFP, MHB and RP

(i) Schematic depicting the normal spatial relationships among the RP (light blue), MFP (dark blue), LFP (green), MHB (dark brown) and dorsal midbrain (beige). **(ii)** Unilateral *SHH* misexpression (right side) ectopically induces LFP and can induce MFP along the MHB while concurrently suppressing MHB fates. Dorsal cell fates, including RP are also suppressed. **(iii)** Unilateral *FOXA2* misexpression can ectopically induce MFP along the MHB as well as away from the MHB. **(iv)** In the absence of HH, the MFP can be converted into the MHB or the RP. **(v)** The loss of FOXA2 converts the ventral midbrain and the MFP into the MHB. As a consequence of ectopic MHB induction, midbrain fate (*OTX2*+) is suppressed (not shown) and hindbrain fate (*GBX2*+, red) is induced. **(vi)** *FOXA2* overexpression combined with HH blockade results in ectopic RP induction throughout ventral midbrain.

Table 1

Summary of gene expression patterns identifying the midbrain signaling centers. Unique identifiers for each signaling center are highlighted in red.

Lateral Floor Plate	SHH	FOXA2								
Medial Floor Plate	SHH	FOXA2	DISP1	MN-CAD	LMX1 A	LMX1B	BMP7			
Midbrain-Hindbrain Boundary						LMX1B	BMP7	WNT1	FGF8	
Roof Plate						LMX1B	BMP7	WNT1		GDF7