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A revised model of *Xenopus* dorsal midline development: Differential and separable requirements for Notch and Shh signaling

Sara M. Peyrot [a,b,1], John B. Wallingford [a,b,c], Richard M. Harland [a,*]

[a] Dept. of Molecular and Cell Biology and Center for Integrative Genomics, University of California, Berkeley, CA 94720, USA
[b] Dept. of Molecular Cell and Developmental Biology, University of Texas, Austin, TX 78712, USA
[c] Howard Hughes Medical Institute, USA

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ABSTRACT

The development of the vertebrate dorsal midline (floor plate, notochord, and hypochord) has been an area of classical research and debate. Previous studies in vertebrates have led to contrasting models for the roles of Shh and Notch signaling in specification of the floor plate, by late inductive or early allocation mechanisms, respectively. Here, we show that Notch signaling plays an integral role in cell fate decisions in the dorsal midline of *Xenopus laevis*, similar to that observed in zebrafish and chick. Notch signaling promotes floor plate and hypochord fates over notochord, but has variable effects on *Shh* expression in the midline. In contrast to previous reports in frog, we find that Shh signaling is not required for floor plate vs. notochord decisions and plays a minor role in floor plate specification, where it acts in parallel to Notch signaling. As in zebrafish, Shh signaling is required for specification of the lateral floor plate in the frog. We also find that the medial floor plate in *Xenopus* comprises two distinct populations of cells, each dependent upon different signals for its specification. Using expression analysis of several midline markers, and dissection of functional relationships, we propose a revised allocation mechanism of dorsal midline specification in *Xenopus*. Our model is distinct from those proposed to date, and may serve as a guide for future studies in frog and other vertebrate organisms.

Introduction

The floor plate (FP) is a specialized population of cells in the ventral portion of the vertebrate neural tube, morphologically and molecularly distinct from other regions of the central nervous system (Kingsbury, 1940; McKanna, 1993). FP cells do not give rise to neurons and adopt a wedge-shaped morphology. The FP expresses *Shh* and *FoxA2* and has signaling activities crucial to neural development (Placzek et al., 1990a,b; Tessier-Lavigne et al., 1988). The FP specifies the identity of neurons along the dorsoventral axis of the spinal cord, an activity mediated by the secreted morphogen Sonic Hedgehog (Shh) (Briscoe and Ericson, 2001; Yamada et al., 1993). In addition, Netrin and F-spondin, two chemoattractants secreted by the FP, function along with Shh to direct commissural axonal guidance (Charron et al., 2003; Klar et al., 1992; Serafini et al., 1996).

Given the critical role of the FP in neural development, it is important to understand how the FP is specified. Several models have been proposed for the development of the FP, as a result of work in different model systems (Le Douarin and Halpern, 2000; Placzek et al., 2000; Strahle et al., 2004). The "induction" model, based on classical embryology experiments in chick and mutant phenotypes in mouse, states that Shh secreted by the notochord induces the formation of the FP in the overlying naïve neurectoderm (Artinger and Bronner-Fraser, 1993; Marti et al., 1995; Placzek et al., 2000, 1993, 1990b; Roelink et al., 1995; Yamada et al., 1991). However, the finding that all midline structures—notochord, FP, and dorsal endoderm—arise at the same time from the organizer in chick indicated that the FP was pre-determined prior to notochord signals, and gave rise to the "allocation" model (Catala et al., 1996, 1995; Teillet et al., 1998).

A "revised allocation" model has been proposed for zebrafish (Odenthal et al., 2000) and supported in chick (Charrier et al., 2002). In zebrafish, the FP consists of two distinct populations, medial (MFP) and lateral (LFP), which are Shh-independent and Shh-dependent, respectively (Etheridge et al., 2001; Odenthal and Nusslein-Volhard, 1998; Odenthal et al., 2000; Schauerte et al., 1998). The MFP is allocated from a midline precursor cell population, which also gives rise to the notochord and dorsal endoderm (hypochord in non-amniotes); Nodal, Notch, and midkine signaling have been implicated in MFP specification (Appel et al., 1999, 2001; Gritsman et al., 1999; Hatta et al., 1991; Muller et al., 2000; Rebagliati et al., 1998; Sampath et al., 1998; Schafer et al., 2005b; Strahle et al., 1997; Tian et al., 2003). The LFP is then induced by Shh secreted from the notochord and MFP (Odenthal et al., 2000).

* Corresponding author at: 571 Life Sciences Addition #3200, University of California, Berkeley, CA 94720, USA. Fax: +1 510 643 6791.
E-mail address: harland@berkeley.edu (R.M. Harland).
[1] Present address: Dept. of Biochemistry, Stanford University, Stanford, CA 94305, USA.

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Despite the long-standing interest in FP development and extensive examination in several animals, comparatively little attention has been paid to this process in *Xenopus*. Early overexpression experiments indicated that induction mechanisms can operate in *Xenopus*, as Shh and FoxA2 can induce other FP markers such as *F-spondin* (Roelink et al., 1994; Ruiz i Altaba et al., 1995), but notably in a temporally and spatially restricted manner unlike endogenous FP development. Importantly, no loss-of-function experiments had been conducted to determine whether Shh signaling is required for FP specification in the frog until recently. Thus, the induction model of FP specification has not been rigorously tested in *Xenopus*.

Because the sheet-like gastrulation movements in frog appear so different from ingression at the node/shield in chick, mouse, and zebrafish, we decided to investigate whether "allocation" mechanisms operate in *Xenopus* FP development. Classical transplantation experiments by Spemann and Mangold (1924), as well as more recent lineage analysis (Bauer et al., 1994; Dale and Slack, 1987; Keller, 1975, 1976), show that the organizer gives rise to dorsal midline structures. This demonstrates the existence of a midline precursor cell (MPC) population in the frog, and therefore the cell lineage aspect of the allocation model. However, little functional analysis has been performed to ascertain what signaling pathways influence the allocation of these cells to midline tissues. Because Notch signaling regulates cell fate choices in the midline in zebrafish (Appel et al., 1999) and chick, (Gray and Dale, 2010), we initially chose to focus on this pathway to test the allocation model in frog. Previous findings on Notch in *Xenopus* midline development (Lopez et al., 2003, 2005) mostly conform to the allocation model proposed by original work on the zebrafish *deltaA* mutant, indicating a high degree of conservation in this process. However, these results in frog do not agree with more recent experiments in zebrafish, which indicate that Notch signaling plays a minor role in FP development (Latimer and Appel, 2006; Schafer et al., 2007).

Given the amphibians' close evolutionary relationship with amniotes, the paucity of data concerning the mechanism of FP specification in these animals, and recent discrepancies between zebrafish and frog on the role of Notch signaling in the midline, we have reexamined midline development in *Xenopus*. We have used both gain- and loss-of-function approaches, and examined both early and definitive markers of FP and notochord. We found that the Notch pathway promotes formation of FP at the expense of notochord, as reported (Lopez et al., 2003, 2005), and that Notch signaling plays a major role in hypochord development in the frog, consistent with data from the zebrafish (Appel et al., 1999; Latimer and Appel, 2006; Latimer et al., 2002). Surprisingly, we find that Shh signaling is not downstream of Notch as previously suggested (Lopez et al., 2003, 2005). Rather, Shh acts in parallel to Notch to contribute modestly to FP specification and has no role in hypochord or notochord specification. Finally, through examination of several gene expression patterns over time, we find unexpected complexity in the development of the *Xenopus* floor plate. We identify an *Nkx2.2*$^+$ LFP as observed in other animals, but we also identify spatially, temporally, and molecularly distinct cell populations in the *Xenopus* MFP. Early MFP cells develop independent of Notch signaling and express *Shh*, while specification of a later population of *Netrin*-expressing MFP cells is Notch-dependent. We have interpreted our results in the context of the revised allocation model of MFP and LFP specification and current research on the roles of Notch and Shh signaling in the development of these structures. We propose a new model for midline specification in *Xenopus* that may serve as a paradigm for further experimentation in other vertebrates.

Materials and methods

General methods and microinjection

Xenopus laevis embryos were generated, microinjected, and cultured by standard methods (Sive et al., 2000). For morpholinos (Gene Tools, LLC), microinjections (10 nL) were performed bilaterally at the 2-cell stage, with 4 ng fluoresceinated standard control morpholino plus Shh morpholinos as follows: Shh splice MO (5′ CACGTATGGCCGTACCTGAGTCATG 3′) 75 ng, and Shh ATG MO (5′ ATCTCGTCCGAGCGAAGCCAATTAC 3′) 50 ng per injection. Synthetic capped mRNAs were produced with the Ambion mMessage machine kit. An alternate expression construct for Shh was made by cloning the HindIII/Spe1 insert of *X-Shh T7TS* (Ekker et al., 1995) into CS2+. This construct (*CS2 + Shh*), as well as *CS2-NLS-MT-NICD* and *CS2-Xsu(H) DBM*, gifts of Dr. C. Kintner (Wettstein et al., 1997), and *CS2 + memEGFP* and *CS2 + βgal*, gifts of Dr. D. Turner, were linearized with Not1 and transcribed with SP6. To target the dorsal midline, mRNA (10 nL) was injected equatorially into both dorsal blastomeres at the 4-cell stage, on either side of the cleavage plane. Embryos were screened by epifluorescence (eGFP) at stage 10 for correct targeting and some were stained for lacZ to lineage trace injected cells. Total embryo doses are as follows: NICD and SDBM, 1 ng; Shh, 400 pg; β-gal and eGFP, 200 pg.

Cyclopamine treatment

A stock solution of cyclopamine (LC Laboratories) was made at 10 mM in 100% ethanol and diluted to 100 μM in 1/3× MR for embryo treatment. Embryos at stage 8–9 were transferred to 1/3× MR + 100 μM cyclopamine or 1/3× MR with an equivalent volume of ethanol (solvent) as a control and allowed to develop to the desired stage, then fixed in MEMFA.

RT-PCR

Whole embryos were lysed and RNA isolated and reverse-transcribed by standard methods (Wills et al., 2009). RNA was prepared from 3 individual embryos for each treatment, then pooled for RT and PCR. For semi-quantitative PCR, PCR was performed with trace [α-^{32}P] dCTP, with primers and conditions as follows:

Shh-splice (fwd) 5′ AGCGGCAGATACGAAGGAAAG
(rev) 5′ TCCCCTCATAATGTAGCGACTCC, 57 °C anneal, 25 cycles
EF1α (fwd) 5′ CAGATTGGTGCTGGATATGC
(rev) 5′ ACTGCCTTGATGACTCCTAG, 55° anneal, 21 cycles.

PCR samples were resolved on polyacrylamide gels and exposed to film.

Whole mount in situ hybridization, immunohistochemistry, and sectioning

Embryos were fixed in MEMFA for 2 h at room temperature or overnight at 4 °C for in situ hybridization (Sive et al., 2000). For lacZ staining, embryos were fixed for 30 min in MEMFA, rinsed in Ptw (1× PBS, 0.1% Tween20), and incubated in lacZ staining solution (5 mM ferricyanide, 5 mM ferrocyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal or Red-gal (Research Organics) in PTw) until the desired color was achieved, then fixed as above for in situ hybridization. Fixed embryos were dehydrated in methanol and stored at −20 °C for further processing. In situ hybridization was performed in baskets as described (Sive et al., 2000) with probes at 1 μg/mL. Plasmids were linearized and transcribed as follows: *AXPC* (Kim et al., 1998) Not1, T7; *chordin* (Sasai et al., 1994) EcoR1, T7; *FoxA4b* (Dirksen and Jamrich, 1992) BamH1, T7; *FoxA4a* (Ruiz i Altaba and Jessell, 1992) Not1, T7; *FoxA1b* (Bolce et al., 1993) EcoR1, T3; *FoxD5a* (Solter et al., 1999) Sma1, T7; *F-spondin* (Klar et al., 1992) Sal1, T7; *FoxA2* (IMAGE clone 8319281) Xma, T7; *Netrin* (de la Torre et al., 1997) Xho1, T3; *Nkx2.2* (Saha et al., 1993) Not1, T7; *Ptc2* (Takabatake et al., 2000) Not1, T7; *Shh* (Ruiz i Altaba et al., 1995) Not1, T3; *VEGF* (Cleaver et al., 1997) BamH1, T7; *Xbra* (Smith et al., 1991) XhoI, SP6; *XencR1* (Haigo et al., 2003) BamH1, T7; and *Xnot* (von Dassow et al., 1993), HindIII, T7. Templates were transcribed as described (Sive et al.,