Essential role for ligand-dependent feedback antagonism of vertebrate hedgehog signaling by PTCH1, PTCH2 and HHIP1 during neural patterning

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SUMMARY
Hedgehog (HH) signaling is essential for vertebrate and invertebrate embryogenesis. In Drosophila, feedback upregulation of the HH receptor Patched (PTC; PTCH in vertebrates), is required to restrict HH signaling during development. By contrast, PTCH1 upregulation is dispensable for early HH-dependent patterning in mice. Unique to vertebrates are two additional HH-binding antagonists that are induced by HH signaling, HHIP1 and the PTCH1 homologue PTCH2. Although HHIP1 functions semi-redundantly with PTCH1 to restrict HH signaling in the developing nervous system, a role for PTCH2 remains unresolved. Data presented here define a novel role for PTCH2 as a ciliary localized HH pathway antagonist. While PTCH2 is dispensable for normal ventral neural patterning, combined removal of PTCH2- and PTCH1-feedback antagonism produces a significant expansion of HH-dependent ventral neural progenitors. Strikingly, complete loss of PTCH2-, HHIP1- and PTCH1-feedback inhibition results in ectopic specification of ventral cell fates throughout the neural tube, reflecting constitutive HH pathway activation. Overall, these data reveal an essential role for ligand-dependent feedback inhibition of vertebrate HH signaling governed collectively by PTCH1, PTCH2 and HHIP1.

KEY WORDS: Neural tube, Hedgehog, Negative feedback

INTRODUCTION
During embryogenesis, complex gene expression patterns arise within fields of initially equivalent cells to form the tissues that comprise a fully developed organism (Perrimon and McMahon, 1999). A small number of conserved signaling pathways act via secreted ligands to establish these embryonic patterns, producing distinct cellular responses in a concentration- and time-dependent manner (Freeman, 2000; Kutejova et al., 2009; Ulloa and Briscoe, 2007). These pathways require precise spatial and temporal control of ligand production and distribution to preserve the requisite diversity of cellular responses and to limit signaling within the appropriate domains. Thus, feedback antagonism of secreted ligands plays a crucial role in regulating the level and spatial distribution of signaling within a target field (Freeman, 2000; Perrimon and McMahon, 1999).

Hedgehog (HH) proteins are secreted molecules that play crucial roles in both vertebrate and invertebrate development (McMahon et al., 2003). Negative feedback at the level of the HH ligand is governed by the canonical receptor Patched (PTC; PTCH in vertebrates) (Chen and Struhl, 1996). Seminal studies in Drosophila identified two distinct forms of PTC-mediated antagonism (Chen and Struhl, 1996). In the absence of ligand, PTC inhibits the activity of Smoothened (SMO) (Ingham et al., 2000), a key effector of the pathway, in a process termed ligand-independent antagonism (LIA) (Jeong and McMahon, 2005). Ligand binding to PTC relieves SMO inhibition and culminates in modulation of HH target genes, including ptc itself. Consequently, PTC is highly upregulated near the source of HH production to bind and sequester ligand and limit the level and the range of signaling within a responding tissue (Hooper and Scott, 1989; Nakano et al., 1989). This negative feedback by PTC at the level of ligand is known as ligand-dependent antagonism (LDA) (Jeong and McMahon, 2005).

Evidence from Drosophila suggests that PTC upregulation is dispensable for SMO inhibition (LIA), but is required to sequester HH ligand and prevent pathway activation in cells more distal to the HH source (LDA) (Chen and Struhl, 1996). Feedback upregulation of the vertebrate PTCH1 receptor is conserved in mammals (Goodrich et al., 1996); however, similar experiments that abrogate PTCH1-feedback upregulation in mice do not dramatically alter HH signaling during early embryogenesis (Jeong and McMahon, 2005; Milenkovic et al., 1999). In this model, tonal levels of PTCH1 are produced from a transgene using the metallothionein promoter (MT-Ptc1) (Milenkovic et al., 1999). In MT-Ptc1;Ptc1−/− embryos, basal levels of PTCH1 are sufficient for LIA, but surprisingly, given the Drosophila studies, these embryos display a largely normal body plan at E10.5, with relatively minor disturbances of HH-dependent patterning (Jeong and McMahon, 2005; Milenkovic et al., 1999).

In contrast to Drosophila, vertebrates possess two additional cell surface HH-binding proteins that are induced by HH signaling: hedgehog interacting protein 1 (HHIP1; HHIP – Mouse Genome Informatics) (Chuang and McMahon, 1999), a membrane-anchored
glycoprotein, and patched 2 (PTCH2; Motoyama et al., 1998b), a structural homolog of PTCH1 that arose from a gene duplication event. HHIP acts partially redundantly with PTCH1 to antagonize HH signaling in the developing mouse central nervous system (CNS); embryos lacking both HHIP-1 and PTCH1-feedback inhibition generate cell fates within the normal HH signaling domain that are more ventral than expected, and HH responses extend into dorsal regions that do not normally exhibit active signaling (Jeong and McMahon, 2005). By comparison, mutant embryos such as Ptch1Δ−/− where LIA is removed, and SufuΔ−/−, where the pathway is activated downstream of ligand, have an even more ventralized phenotype with a significant extent of the neural tube adopting a Sonic HH (SHH)-secreting floorplate fate (Cooper et al., 2005; Goodrich et al., 1997). The differences in the severity of these patterning defects suggest that other mechanisms limit ligand-dependent vertebrate HH pathway activity.

The contribution of PTCH2 in feedback antagonism during CNS patterning has not been addressed. Mouse PTCH1 and PTCH2 share 56% amino acid identity; a key difference is that PTCH2 is more stable than PTCH1 due to a truncated C-terminal region (Kawamura et al., 2008). Although PTCH2 antagonizes HH signaling in cell-based assays (Rahnamai et al., 2004), Ptch2Δ−/− mice are viable and fertile, whereas Ptch1Δ−/− mice die at E9.5 with ectopic HH signaling throughout the embryo (Goodrich et al., 1997; Nieuwenhuis et al., 2006). Ptch2Δ−/− embryos do, however, exhibit subtle changes in gene expression that are consistent with increased HH pathway activation, including a slight expansion of Ptch1 and Gli1 expression in the developing limb bud and the embryonic hair follicle (Nieuwenhuis et al., 2006). These transcriptional changes ultimately resolve to produce normally patterned HH-responsive tissues, although aged male Ptch2Δ−/− and Ptch2Δ−/− mice develop epidermal hyperplasia and alopecia (Nieuwenhuis et al., 2006). That PTCH1 action may mask PTCH2 activity is a reasonable hypothesis, especially given the observation that the loss of PTCH1 action may mask PTCH2 activity.

In the developing mouse neural tube, complete loss of PTCH2-, HHIP1- and PTCH1-feedback inhibition results in a neural tube composed entirely of ventral cell populations. In addition, complete loss of PTCH2- and PTCH1-feedback inhibition results in a neural tube composed entirely of ventral cell populations, similar to Ptch1Δ−/− in the developing mouse CNS; embryos lacking both HHIP1- and PTCH1-feedback inhibition results in a neural tube composed entirely of ventral cell populations. Although embryos lacking PTCH2 alone or in combination with HHIP1 display normal neural patterning, combined loss of PTCH2- and PTCH1-feedback inhibition results in a significant expansion of SHH-dependent ventral cell populations. In addition, complete loss of PTCH2-, HHIP1- and PTCH1-feedback inhibition results in a neural tube composed entirely of ventral cell populations, similar to Ptch1Δ−/− and SufuΔ−/− embryos. Overall, these data demonstrate an essential role for negative feedback at the level of HH ligand during vertebrate development, and reveal a collective requirement for PTCH1, PTCH2 and HHIP1 in ligand-dependent feedback inhibition.

**MATERIALS AND METHODS**

**Mice**

Ptch2 mice were generated and provided by Curis. The absence of Ptch2 mRNA was confirmed by expression analysis in the testes, the highest site of Ptch2 expression (Carpen ter et al., 1998). Hhip1 (Chuang et al., 2003), Ptch1 (Goodrich et al., 1997) and MtPtch1 (Milenkovic et al., 1999) mice have all been previously described. For timed pregnant analyses, noon of the day on which a vaginal plug was detected was considered E0.5. Precise embryo staging was achieved by assessment of somite number at the time of dissection. For each analysis, a minimum of three embryos were analyzed and representative images are shown.

For transgenic analysis of the Ptch2 enhancer, the Ptch2 enhancer region (chr11:116,768,296-116,768,754) was PCR amplified from C57Bl/6J genomic DNA, sequence verified and cloned upstream of a modified Hsp68-lacZ reporter construct containing a single copy of the chicken β-globin insulator. Transient transgenics were generated via pronuclear injection and collected at E10.5. PCR genotyping and X-gal staining were performed as previously described (Vokes et al., 2007).

**Chick in ovo neural tube electroporations**

Electroporations were performed as previously described (Allen et al., 2011). In brief, DNA (1.0 μg/μl) was injected into the neural tubes of Hamburger-Hamilton stage 10-12 chicken embryos with 50 ng/μl Fast Green. Embryos were dissected after 48 hours and fixed in 4% PFA for immunofluorescent analysis.

**Immunofluorescence**

Immunofluorescence was performed essentially as previously described (Allen et al., 2011). Neural patterning analysis was performed at the forelimb level in E9.5 and E10.5 embryos. The following antibodies were used: mouse IgG1 anti-NKX6.1 [1:20, Developmental Studies Hybridoma Bank (DSHB)], mouse IgG2a anti-PAX3 [1:20, DSHB], rabbit IgG anti-DBX1 [1:1000, gift from Dr Yasushi Nagakawa, University of Minnesota, Minneapolis, MN, USA], mouse IgG1 anti-FOXA2 [1:20, DSHB], rabbit IgG anti-FOXA2 [1:500, Cell Signaling], mouse IgG2b anti-NKX2.2 [1:20, DSHB], rabbit IgG anti-OLIG2 [1:1000, Millipore], mouse IgG1 anti-SHH [1:20, DSHB], rabbit IgG anti-cleaved caspase 3 [1:200, Cell Signaling), rabbit IgG anti-phospho-histone H3 [1:1000, Millipore], mouse IgG1 anti-MNR2 [1:20, DSHB], mouse IgG2b anti-ISL1 [1:20, DSHB], mouse IgG2a anti-EVX1 [1:20, DSHB] and mouse IgG1 anti-EN1 [1:20, DSHB]. Nuclei were visualized using DAPI [1:30,000, Molecular Probes]. Alexa 488, 555 and 633 secondary antibodies (1:500, Molecular Probes) were visualized on a Leica upright SP5X confocal microscope.

**Cellular localization of HH pathway components**

NII/3T3 fibroblasts were plated at 150,000 cells/well on coverslips and transfected 16-24 hours later. Six hours post-transfection, cells were placed into low-serum (0.5%) media and fixed 48 hours later in 4% PFA for immunofluorescent analysis.

**Luciferase assays**

Luciferase assays were adapted from a previously published protocol (Nybakken et al., 2005). Mouse NIH/3T3 fibroblasts were plated at 25,000 cells/well on gelatinized 24-well plates and transfected 16-24 hours later with 150 ng of a pC1A136-G3 luciferase reporter (Chen et al., 1999; Nybakken et al., 2005), 50 ng of pSv-p-galactosidase (Promega) and 100 ng of control (pcGIG) or experimental constructs using Lipofectamine 2000 (Invitrogen). After 48 hours, cells were placed in low serum (0.5%) media with 25 μl of conditioned media from control (pCDNA3) or NSHH transfected (NSHr-pCDNA3) COS7 cells. Luciferase (Luciferase Assay System Kit, Promega) and β-galactosidase (β-galactosidase assay Kit, Novagen) activity were measured after 48 hours. Luciferase values were normalized to β-galactosidase activity and expressed as fold induction relative to control treated cells. Signaling assays in Ptch1Δ−/− MEFs (a gift from Dr. M. P. Scott, Stanford University, CA, USA) were performed as described with the following modifications. Ptch1Δ−/− MEFs were plated at 50,000 cells per well and secreted placental alkaline phosphatase was used as a transfection control (Alkaline Phosphatase Yellow pNPP Liquid Substrate for ELISA, Sigma-Aldrich).

**Immunoprecipitation**

COS7 cells were plated at 1×10⁶ cells/100 mm dish and transfected the next day with expression plasmids for the indicated proteins. Immunoprecipitations and western blot analyses were performed as previously described (Okada et al., 2006). In brief, PTCH-FA proteins were immunoprecipitated using a mouse anti-HA antibody (SIGMA H3663). Western blot analyses were then performed using mouse anti-HA, rabbit anti-GFP (Molecular Probes A11122), goat anti-Gas1 (R&D AF2644) and mouse anti-Axin (SIGMA A5441) to reveal the input and IP levels.

**In situ hybridization**

In situ hybridization was performed essentially as described previously (Wilkinson, 1992) using digoxigenin-labeled probes on 20 μm sections collected at the forelimb level of E9.5 and E10.5 embryos.
RESULTS

**Ptch2 is a direct HH target that antagonizes SHH-mediated pathway activation in vivo**

Genomic characterization of GLI1- and GLI3-binding profiles highlight HH pathway components, including *Ptch1*, *Ptch2* and *Hhip1*, as direct transcriptional targets of HH signaling (Peterson et al., 2012; Vokes et al., 2007; Vokes et al., 2008). Previously, a promoter proximal GLI1-binding region (GBR) associated with *Ptch1* recapitulated the majority of *Ptch1* expression, including in the CNS (Vokes et al., 2007). Similarly, *Ptch2* possesses a conserved promoter proximal GBR (*Ptch2*<sup>−0.5kb</sup>; Fig. 1A); however, the regulatory potential of this region remains unexplored. To determine the enhancer activity associated with *Ptch2*<sup>−0.5kb</sup>, we isolated a highly conserved 459 bp region and assayed reporter expression in transient transgenic mouse embryos at E10.5 (Fig. 1B). The *Ptch2*<sup>−0.5kb</sup> enhancer displays a ventral distribution throughout the entire CNS, consistent with *Ptch2* as a direct readout of SHH signaling (Fig. 1C,D) and in line with previous reports of *Ptch2* expression in the neural tube (Motoyama et al., 1998a).

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**Fig. 1. PTCH2 is a direct transcriptional target that antagonizes HH signaling in NIH/3T3 cells and in the developing chick neural tube.**

(A) *Ptch2* regulatory landscape highlighting two discrete GLI1 binding events positioned at −0.5 kb and +2.2 kb relative to TSS. (B) Higher magnification view of −0.5 kb region assayed for enhancer activity (blue bar). Computationally predicted GLI-binding sites (GBS) are shown in black (nonconserved) and red (conserved). Multi-species conservation (cons.) is shown below. (C) Transient transgenic analysis of *Ptch2*<sup>−0.5kb</sup> regulatory region shows neural-specific activity at E10.5. The number of embryos expressing the transgene out of total transgenic positives is shown in the upper right-hand corner. (D) Transverse section taken from region indicated in C (black bar) shows reporter activity restricted to the ventral neural tube. (E) HH-responsive luciferase reporter activity measured in NIH/3T3 fibroblasts stimulated with either control media (white bars) or NSHH-conditioned media (grey bars), and co-transfected with the indicated constructs. Each condition was performed in triplicate and data are represented as mean±s.e.m., *P*-values measured by two-tailed Student’s *t*-test. (F-U) Hamburger-Hamilton stage 19-22 chick neural tubes electroporated with *pCIG* (F-I), *Ptch1::HA-pCIG* (J-M), *Ptch2::HA-pCIG* (N-Q) and *Ptch1ΔL2::HA-pCIG* (R-U) sectioned at the wing level and stained with antibodies raised against NKX6.1 (red, F,G,J,K,N,O,R,S) or PAX7 (red, H,I,L,M,P,Q,T,U). Nuclear EGFP expression (G,I,K,M,O,Q,S,U) labels electroporated cells. Arrows indicate repression of NKX6.1 expression (J,K,N,O,R,S) or ectopic expression of PAX7 (L,M,P,Q,T,U). Arrowheads indicate ventrally located electroporated cells that maintain NKX6.1 expression (J,K,N,O) or lack ectopic PAX7 expression (L,M,P,Q). Scale bar: 50 μm in F-U.
To examine PTCH2-mediated antagonism of HH signaling, and to compare PTCH2 directly with other cell surface HH pathway antagonists, we expressed PTCH2, PTCH1 and HHIPI in HH-responsive NIH3T3 fibroblasts. Cells transfected with a HH-responsive luciferase reporter (Chen et al., 1999) and treated with SHH show robust induction of luciferase activity compared with untreated cells (Fig. 1E). Consistent with previous reports, expression of PTCH1, HHIPI or PTCH2 inhibits NSHH-mediated pathway activation (Fig. 1E) (Rahnana et al., 2004).

To extend these results, we used chick in ovo electroporations to determine whether PTCH2 could antagonize SHH-dependent ventral neural patterning. During nervous system development, a gradient of SHH directly induces (class II genes Nkx6.1, Nkx2.2, Foxa2, etc.) or indirectly represses (class I genes Pax7, Pax5, etc.) expression of a series of transcriptional regulators in a concentration-dependent manner (Dessaud et al., 2008). The combinatorial activity of these transcriptional determinants along the dorsal-ventral (DV) axis specifies unique neural progenitor domains that generate distinct classes of mature neurons (Briscoc et al., 2000). These targets quantitatively readout HH pathway activity in vivo.

Ectopic expression of EGFP (pCIG) in the chick neural tube does not affect neural patterning (Fig. 1F-I) based on expression of Nkx6.1 (class II target) and Pax7 (class I target). By contrast, overexpression of either PTCH1 or PTCH2 represses Nkx6.1 (Fig. 1J,K,N,O, arrows) and de-represses Pax7 expression in the ventral neural tube (Fig. 1L,M,P,Q, arrows), consistent with PTCH1 (Fig. 1J,K,N,O, arrows) and de-represses Pax7 expression in the ventral neural tube (Fig. 1L,M,P,Q, arrows). Consistent with PTCH1 and PTCH2 acting as HH pathway antagonists. Importantly, Nkx6.1 is maintained and Pax7 remains repressed in more ventral cells electroporated with either PTCH1 or PTCH2 (Fig. 1J-Q, arrowheads), suggesting that patched-mediated antagonism can be overcome by higher ligand concentrations. By contrast, a ligand-insensitive PTCH1 (PTCH1ΔL) that acts as a constitutive SMO antagonist (Briscoc et al., 2001) inhibits Nkx6.1 and enables Pax7 expression in a position-independent manner (Fig. 1R-U). Taken together, these data are consistent with PTCH2 acting as a HH pathway antagonist.

**Ptch2−/− and Ptch2−/−;Hhip1−/− embryos display normal ventral neural patterning**

To determine the endogenous actions of PTCH2, we analyzed SHH-dependent ventral neural patterning in Ptch2−/− embryos. Consistent with other published alleles, Ptch2−/− mice are viable and fertile (Nieuwenhuis et al., 2006) and display a grossly normal body plan at E10.5 (supplementary material Fig. S2A,B). As reported for Hhip1 mutants (Jeong and McMahon, 2005), Ptch2−/− embryos exhibit no overt defects in ventral neural patterning at E10.5 (Fig. 2B,G; supplementary material Fig. S1).

To address possible redundancy between PTCH2 and HHIPI functions, we analyzed neural patterning in Ptch2;Hhip1 double mutant embryos at E10.5 (Fig. 2); however, Ptch2−/−;Hhip1−/− embryos are grossly normal and exhibit no defects in ventral neural patterning at this stage (Fig. 2E,J; supplementary material Fig. S2C).

**Expansion of ventral neural progenitors in embryos lacking PTCH1 and PTCH2-feedback antagonism**

Given the previously identified redundancy between PTCH1 and HHIPI, we reasoned that PTCH1-feedback inhibition is sufficient to antagonize SHH signaling in Ptch2;Hhip1 double mutants. Thus, to uncover a role for PTCH2, we used an MT-PITCH1 transgene that produces sufficient levels of PTCH1 for LIA of SMO (Milenkovic et al., 1999) in order to compare the phenotypes of MT-Pitch1/Pitch1−/− embryos that lack PTCH1-mediated LDA with MT-Pitch1/Pitch1−/−;Pitch2−/− embryos, which are incapable of both PTCH1 and PTCH2-dependent LDA.

As previously reported, MT-Pitch1/Pitch1−/− embryos display a grossly normal body plan at E10.5 (supplementary material Fig. S2D) (Jeong and McMahon, 2005; Milenkovic et al., 1999), although a subtle expansion of ventral cell identities is detected when compared with wild-type embryos at E10.5 (Fig. 3A,B,E,F). This is in stark contrast to analogous experiments performed in Drosophila, where removal of PTC-feedback inhibition completely abrogates receptor-mediated feedback antagonism (Chen and Struhl, 1996). Intriguingly, MT-Pitch1/Pitch1−/−;Pitch2−/− embryos exhibit midbrain and hindbrain exencephaly (supplementary material Fig. S2E) – similar to mutants lacking GLI3 repressor activity (Hui and Joyner, 1993) and consistent with overactive HH pathway activity. Compared with MT-Pitch1/Pitch1−/− embryos, MT-Pitch1/Pitch1−/−;Pitch2−/− embryos also exhibit significant expansion of SHH-dependent Nkx6.1 (Fig. 3D), FoxA2, Nkx2.2 and Olig2 (Fig. 3H) expression at E10.5, which is indicative of an increased range of HH signaling in the absence of PTCH2. In particular, Nkx2.2+ cells, which require a high threshold for induction (Ericson et al., 1997), are dorsally extended in MT-Pitch1/Pitch1−/−;Pitch2−/− embryos (Fig. 3I, arrows, see inset). The ventral expansion is accompanied by retraction of the dorsal Pax3+ domain in MT-Pitch1/Pitch1−/−;Pitch2−/− embryos (Fig. 3D). Quantitatively demonstrates a significant increase in FoxA2+ floorplate cells (Fig. 3I), Nkx2.2+ v3 interneuron progenitors (Fig. 3J) and the proportion of the neural tube that is Nkx6.1+ in embryos (Fig. 3C,H).
**Inhibition of HH signaling**

**RESEARCH ARTICLE**

**Fig. 3. Expansion of SHH-dependent ventral progenitor domains in E10.5 mouse embryos lacking both PTCH2- and PTCH1-feedback antagonism.** (A-H) Neural patterning analysis in E10.5 forelimb sections using antibodies against NKX6.1, DBX1, PAX3 (red, green and magenta, respectively; A-D), FOXA2, NKX2.2 and OLG2 (red, green and magenta, respectively; E-H) in wild type (A,E), MT-Ptch1;Ptch1<sup>+/−</sup>;Ptch2<sup>+/−</sup> (B,F), MtPtch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup> (C,G) and MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup> (D,H) embryos. Insets show NKX2.2 channel alone (E-H). Arrows indicate dorsal expansion of NKX2.2<sup>+</sup> cells in MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup> embryos (H). (I-K) Quantitation of FOXA2<sup>+</sup> cell number (I), NKX2.2<sup>+</sup> cell number (J) and NKX6.1 domain size as a % of total DV neural tube length (K). Data are represented as mean ± s.e.m. calculated from at least three embryos per genotype. *P*-values are determined by two-tailed Student’s *t*-test. Scale bars: 50 μm.

**MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup> embryos compared with MT-Ptch1;Ptch1<sup>−/−</sup> animals (Fig. 3K).**

These results, in combination with previous studies (Jeong and McMahon, 2005), demonstrate that PTCH2 and HHIP1 functionally compensate for the absence of PTCH1-feedback inhibition during ventral neural patterning. To determine whether transcriptional upregulation of Ptch2 or Hhip1 occurs in the absence of PTCH1-mediated LDA, we examined Ptch2 and Hhip1 expression patterns in the neural tube of MT-Ptch1;Ptch1<sup>−/−</sup> embryos using RNA in situ hybridization (supplementary material Fig. S3). Both Hhip1 and Ptch2 are transcriptionally upregulated in the ventral neural tube of MT-Ptch1;Ptch1<sup>−/−</sup> compared with wild-type embryos at E9.5 and E10.5 (supplementary material Fig. S3). We also observe significant upregulation of Hhip1 transcripts in the paraxial mesoderm in embryos lacking PTCH1-feedback inhibition (supplementary material Fig. S3M-P).

Together, the data suggest that PTCH1, PTCH2 and HHIP1 all contribute to LDA of SHH signaling. Furthermore, when PTCH2 or HHIP1 is absent, the normal patterning response is dependent on PTCH1-mediated LDA.

**Severe neural tube ventralization in E10.5 embryos lacking combined PTCH1-, PTCH2- and HHIP1-feedback antagonism.**

In both MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup> and MT-Ptch1;Ptch1<sup>−/−</sup>;Hhip1<sup>−/−</sup> embryos, a persistent PAX3<sup>+</sup>; NKX6.1<sup>−</sup> dorsal domain suggests that SHH signaling is largely absent from the dorsal neural tube (Fig. 3, Fig. 4B,J). The cells in this region are HH responsive, as evident from Ptch1<sup>−/−</sup> embryos where NKX6.1 extends the length of the DV axis and only a small number of PAX3<sup>+</sup> cells remain (Goodrich et al., 1997). This disparity could be explained by the residual functions of HHIP1 or PTCH2 in MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup> or MT-Ptch1;Ptch1<sup>−/−</sup>;Hhip1<sup>−/−</sup> embryos, respectively.

To test this, we reduced the gene dose of Hhip1 in MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup> embryos. Consistent with this view, MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup>;Hhip1<sup>−/−</sup> embryos display a more severe expansion of ventral cell populations than MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup> embryos (Fig. 4B,C,F,G). Additionally, MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup>;Hhip1<sup>−/−</sup> embryos exhibit a further expansion of ventral cell identities compared with MT-Ptch1;Ptch1<sup>−/−</sup>;Hhip1<sup>−/−</sup> embryos (Fig. 4J,K,N,O). Thus, both HHIP1 and PTCH2 play significant roles when PTCH1/PTCH2 or PTCH1/HHIP1 feedback responses are removed, respectively. Of note, there is significant variability in the degree of patterning defects in these embryos, which likely reflects the large effects from fluctuations in near-threshold levels of dorsal SHH signals (Fig. 4K,O, insets). Interestingly, we also observed significant mixing among different cell populations, indicating that LDA is essential to generate discrete boundaries between progenitor domains (Fig. 4F,G, arrows).

These data support the notion that PTCH1, PTCH2 and HHIP1 together comprise a feedback network of cell surface HH antagonists. To test this hypothesis, we generated embryos that completely lack cell surface feedback antagonism (MT-Ptch1;Ptch1<sup>−/−</sup>;Ptch2<sup>−/−</sup>;Hhip1<sup>−/−</sup>). Grossly, triple mutant embryos display severe exencephaly throughout most of the anterior-posterior axis, CNS overgrowth,
craniofacial abnormalities and enlarged somites (supplementary material Fig. S2G). Remarkably, MT-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− embryos exhibit neural patterning defects comparable with those described in Ptch1−/− embryos (Fig. 4D,H,L,P) (Goodrich et al., 1997): a small dorsal midline cluster of PAX3+ cells, NKX6.1 expression along the entire DV axis (Fig. 4D,L), OLIG2+ motoneuron progenitors confined to the dorsal neural tube, and NKX2.2+ v3 progenitors extending to the dorsal limits of the neuraxis (Fig. 4H,P; supplementary material Fig. S4).

FOXA2 is crucial for induction of SHH at the ventral midline and its activation there requires the highest level of HH signaling (Ribes et al., 2010; Roelink et al., 1995). In MT-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− embryos, FOXA2 production extends throughout the DV axis with high levels of expression ventrally and low levels dorsally (Fig. 4H,P; supplementary material Fig. S4), resulting in a dramatically enlarged SHH-producing floorplate (Fig. 4Q-U). However, persistent NKX2.2+ expression in these cells demonstrates incomplete floorplate maturation (Fig. 4H,P; supplementary material Fig. S4).

As an expected outcome of progenitor misspecification, we also observed a severe reduction in post-mitotic descendants of specific progenitor classes in the absence of all LDA, including motoneurons (ISL1 and MNR2), V1 interneurons (EN1) and V0 interneurons (EVX1; supplementary material Fig. S5). Overall, these data demonstrate a collective requirement for PTCH2-, HHIP1- and PTCH1-feedback inhibition to restrict HH signaling in order to ensure the appropriate diversity of both ventral and dorsal neural progenitor types.

Beyond the severe neural patterning defects observed in the embryos, we also detected significant deficits in the size and cellularity of MT-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− neural tubes, as well as aberrant neuro-epithelial outgrowths (supplementary material Fig. S6A-E, arrows) and cells budding off of the epithelium into the lumen (supplementary material Fig. S6F-J, arrowheads). These morphological defects are accompanied by reduced proliferation and increased apoptosis, as assessed by phospho-histone H3 (PH3) and cleaved caspase 3 (CC3) staining, respectively, in triple mutants (supplementary material Fig. S7).

Notably, apoptotic cells are most prominent in the paraxial mesoderm surrounding the neural tube (supplementary material Fig. S7) and the distal extent of apoptotic mesodermal cells from the notochord increases with the severity of the LDA mutations, suggesting that the cell death is dependent on SHH ligand. Additionally, the loss of paraxial mesoderm could contribute to the lack of mature neurons (supplementary material Fig. S5) owing to compromised retinoic acid production from the somites, which is required for neuronal differentiation in the neural tube (Diez del Corral et al., 2003; Novitch et al., 2003; Sockanathan et al., 2003).

Fig. 4. Severe neural tube ventralization in E10.5 MT-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− embryos. (A-U) Antibody detection of NKX6.1, DBX1, PAX3 (red and green and magenta, respectively; A-D,H,L), FOXA2, NKX2.2, OLIG2 (red, green and magenta, respectively; E-H,M-P), SHH (SE1) and FOXA2 (green and red, respectively; Q-U) in E10.5 forelimb sections from wild type (A,E,J,M,Q), MT-Ptch1;Ptch1−/− (R), MT-Ptch1;Ptch1−/−;Ptch2−/− (B,F,S), MT-Ptch1;Ptch1−/−;Hhip1−/− (J,N,T), MT-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− (C,G), MT-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− (K,O) and MT-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− (D,H,L,P) embryos. Arrows indicate NKX2.2+ cells within the OLIG2 domain (F) and OLIG2+ cells in the NKX2.2 domain (G). Insets are representative of less severe phenotypes that are observed in MT-Ptch1;Ptch1−/−;Hhip1−/−;Ptch2−/− embryos (K,O). Scale bars: 50 μm.

RESEARCH ARTICLE
Severe neural tube ventralization in E8.5 MT-Ptch1;Ptch1+/−;Ptch2−/−;Hhip1−/− embryos is independent of floorplate-derived SHH

The extended SHH domain in MT-Ptch1;Ptch1+/−;Ptch2−/−;Hhip1−/− embryos raises the possibility that the observed patterning defects are secondary to enhanced SHH ligand production rather than due to a direct deficiency of LDA. To resolve these conflicting interpretations, we examined neural patterning at E8.5, where SHH-dependent ventral patterning derives solely from notochord-expressed ligand. Although FOXA2 expression begins around the 8-somite stage, floor-plate SHH expression does not initiate until the 16-somite stage (Jeong and McMahon, 2005). Thus, patterning defects prior to this time reflect direct readouts of the loss of LDA uncomplicated by ectopic SHH from an expanded floorplate.

In Ptch2−/−;Hhip1−/− embryos at E8.5, neural patterning is normal, as indicated by the dorsal restriction of PAX3 and by NKX6.1 expression in the ventral neural tube (Fig. 5A,F). In addition, FOXA2 expression initiates in the ventral midline with normal, as indicated by the dorsal restriction of PAX3 and by NKX6.1 expression limited to the notochord (Fig. 5K). As expected, addition, FOXA2 expression initiates in the ventral midline with normal, as indicated by the dorsal restriction of PAX3 and by NKX6.1 expression limited to the notochord (Fig. 5K). However, FOXA2 expression begins around the 8-somite stage, floor-plate SHH expression does not initiate until the 16-somite stage (Jeong and McMahon, 2005). Thus, patterning defects prior to this time reflect direct readouts of the loss of LDA uncomplicated by ectopic SHH from an expanded floorplate.

Interestingly, the magnitude of this early difference is more marked than that at E10.5, with FOXA2 expression at the dorsalmost region of the neural tube in E8.5 MT-Ptch1;Ptch1+/−;Ptch2−/− and MT-Ptch1;Ptch1+/−;Ptch2−/−;Hhip1+/− embryos (Fig. 5M,N, arrows), a phenotype never observed at E10.5. In some instances, we also observed reduced PAX3 (Fig. 5L) and induction of FOXA2 (Fig. 5M,N) in the somites of LDA mutants at E8.5, suggesting that ligand-mediated feedback antagonism also functions to restrain HH signaling in other HH-responsive tissues. Finally, MT-Ptch1;Ptch1+/−;Ptch2−/−;Hhip1−/− embryos exhibit severe neural tube ventralization that is nearly indistinguishable from Ptch1−/− embryos at E8.5 (Fig. 5J,O). In each instance, immunostaining for SHH confirmed that the patterning defects arise solely from notochord-derived ligand (Fig. 5K-O). Collectively, these results are consistent with a direct requirement for LDA in neural progenitors to limit HH signaling at the onset of ventral neural patterning.

**PTC2 is a ciliary-localized SMO antagonist**

Although the ectopic signaling observed in MT-Ptch1;Ptch1+/−;Ptch2−/−;Hhip1−/− embryos is likely ligand dependent, loss of inhibition downstream of ligand could also contribute to this phenotype. To address this, we tested whether PTCH1, PTCH2 or HHIP1 could antagonize signaling downstream of SMO. In NIH3T3 cells, co-transfection of PTCH1, PTCH2 or HHIP1 with constitutively active SMOM2 (Xie et al., 1998) does not reduce HH pathway activity compared with cells transfected with SMOM2 alone (Fig. 6A); thus, these cell surface molecules act upstream of SMO.

We next explored whether PTCH1, PTCH2 and HHIP1 can directly antagonize SMO activity (LIA). To achieve this, we employed mouse embryonic fibroblasts (MEFs) isolated from Ptch1−/− embryos that lack LIA and exhibit high levels of HH signaling. Although PTCH1 can directly inhibit SMO, there are conflicting reports concerning LIA by PTCH2 (Nieuwenhuis et al., 2006; Rahnama et al., 2004). As previously reported, expression of PTCH1 in Ptch1−/− MEFs causes robust inhibition of HH-responsive luciferase reporter activity even at low concentrations of transfected DNA (Fig. 6B) (Taipale et al., 2002). Although PTCH1 and PTCH2 function equivalently at high DNA concentrations, PTCH2 displays significantly reduced activity at lower concentrations, even though PTCH2 protein is highly stable compared with PTCH1 (Fig. 6B; Kawamura et al., 2008). By contrast, HHIP1 is unable to inhibit SMO at any DNA concentration tested (Fig. 6B). Overall, these results suggest that PTCH2 is capable of LIA of SMO but that PTCH2 activity is weaker than that of PTCH1.

PTCH1 and PTCH2 are structurally related to the RND permease superfamily, which consists of 12-pass transmembrane proteins that function by proton-antiport to efflux small molecules across lipid bilayers (Tseng et al., 1999). This transporter activity is dependent on a conserved RND domain, and missense mutations within the PTCH1 RND motif result in impaired LIA, consistent with PTCH1 functioning catalytically as an RND transporter (Tseng et al., 1999). As previously reported, expression of PTCH1 in Ptch1−/− embryos raises the possibility that the observed patterning defects prior to this time reflect direct readouts of the loss of LDA uncomplicated by ectopic SHH from an expanded floorplate.

**Fig. 5. Expansion of ventral progenitor domains occurs prior to floorplate expression of SHH in E8.5 LDA mutants.** (A-O) DAPI staining (A-E) and neural patterning analysis of E8.5 embryos (9-12 somites) detects expression of NKX6.1 and PAX3 (red, green, respectively; F-J), and FOXA2 and SHH (red, green, respectively; K-O) in Ptch2+/−;Hhip1−/− (EK), MT-Ptch1;Ptch1+/− (GL), MT-Ptch1;Ptch1−/−;Ptch2−/− (H,M), MT-Ptch1;Ptch1−/−;Ptch2+/−;Hhip1−/− (JN) and MT-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− (J,O) embryos. Arrows indicate FOX2A expression at the dorsalmost region of the neural tube (MN). Scale bars: 50 μm.
et al., 1999; Taipale et al., 2002). To determine whether LIA by PTCH2 involves a similar catalytic activity, we generated two analogous PTCH2 RND mutants: PTCH2G465V and PTCH2D469Y. As previously reported, PTCH1G495V and PTCH1D499Y exhibit reduced ability to inhibit SMO in Ptch1−/− MEFs (Fig. 6C). Similarly, PTCH2G465V and PTCH2D469Y display impaired LIA at all concentrations tested (Fig. 6D), consistent with PTCH2 functioning as an RND permease. PTCH2 binds all three mammalian HH ligands with similar affinity as PTCH1 (Carpenter et al., 1998); however, previous work suggested that PTCH2-mediated inhibition of SMO is only relieved after treatment with desert hedgehog (DHH) ligand and does not respond to SHH (Rahnama et al., 2004). We next determined whether PTCH2 could respond to SHH ligand. Interestingly, both PTCH1- and PTCH2-mediated inhibition of SMO is partially relieved upon treatment with SHH (Fig. 6E), suggesting that PTCH2 is responsive to SHH ligand. By contrast, a ligand-insensitive PTCH1 construct (PTCH1ΔL2) is refractory to SHH treatment (Fig. 6E).

During vertebrate embryogenesis, the HH co-receptors GAS1, CDON and BOC are collectively required to initiate HH ligand-mediated responses (Allen et al., 2011). Gas1;Cdon;Boc triple mutant embryos are nearly identical to Smo−/− mutants (Allen et al., 2011), yet HH signaling can be activated downstream of ligand
using small-molecule SMO agonists in co-receptor deficient cerebellar granule neuron precursors (Izzi et al., 2011). Consistent with their role in mediating HH ligand-dependent signaling, GAS1, CDON and BOC interact with PTCH1 and can form distinct receptor complexes (Bae et al., 2011; Izzi et al., 2011). Based on the ability of PTCH2 to respond to SHH, we assessed whether PTCH2 interacts with the HH co-receptors by co-immunoprecipitation. Similar to PTCH1, HA-tagged PTCH2 interacts with GAS1, CDON and BOC in COS7 cells (Fig. 6F), suggesting that PTCH2 can also form complexes with the HH co-receptors.

SMO transduces the HH signal at the primary cilium, an organelle crucial for vertebrate HH signal transduction (Corbit et al., 2005; Huangfu et al., 2003). In the absence of ligand, PTCH1 localizes to the primary cilium to prevent SMO ciliary accumulation and activation. Ligand-binding to PTCH1 delocalizes LIA from the ciliary membrane, enabling downstream signaling through SMO (Rohatgi et al., 2007). To determine whether PTCH2 and HHIP1 also localize to the primary cilium, we expressed HA-tagged PTCH2 and HHIP1 in NIH/3T3 cells to examine co-labeling of HA with the ciliary marker, acetylated tubulin (ACTUB). Consistent with previous studies, PTCH1 localizes to the primary cilium (Fig. 6G-I). Strikingly, we also detect PTCH2 within the ciliary membrane of transfected cells (Fig. 6J-L). This localization is not dependent on a physical interaction with endogenous PTCH1, as PTCH2::HA also localizes to the primary cilium in Ptch1−/−;MEFs (Fig. 6J-L, insets). By contrast, HHIP1::HA does not localize to the primary cilium (Fig. 6M-O). Taken together, these data suggest that ciliary localization is a shared feature between PTCH1 and PTCH2, but that ciliary localization is not a universal requirement for ligand-dependent HH pathway antagonism.

**DISCUSSION**

**A novel role for PTCH2 as a HH pathway antagonist during vertebrate neural patterning**

Although initial studies suggested that PTCH2 plays little to no role in antagonizing HH signaling in vivo (Nieuwenhuis et al., 2006), data presented here support an important role for PTCH2 in restricting HH pathway activity during vertebrate embryogenesis. First, embryos lacking both PTCH1 and PTCH2 feedback inhibition (Mt-Ptch1;Ptch1−/−;Ptch2−/−) display more severe patterning defects than those lacking only PTCH1 feedback antagonism (Mt-Ptch1;Ptch1−/−). This inhibitory role is most evident at E8.5, when HH-dependent ventral patterning initially occurs unopposed by antagonistic roof plate signals, including Wnts and BMPs (Dudley and Robertson, 1997; Parr et al., 1993). Second, the severe ventralization observed in Mt-Ptch1;Ptch1−/−;Ptch2−/−;Hhip1−/− embryos likely results from loss of inhibition at the level of HH ligand (LDA) and not simply from loss of SMO inhibition (LIA). First, only PTCH1 and PTCH2 are capable of LIA; thus, the lack of ectopic signaling in the dorsal neural tube of Mt-Ptch1;Ptch1−/−;Ptch2−/− embryos confirms that PTCH1 levels provided by the Mt-Ptch1 transgene are sufficient for LIA. As HHIP1 is incapable of LIA, the severe ventralization observed upon further removal of HHIP1 likely results from enhanced ligand-dependent signaling. That HHIP1 is indirectly required for LIA is unlikely due to the normal patterning observed in Hhip1−/− and Ptch2−/−;Hhip1−/− embryos. Collectively, these results suggest an essential role for negative feedback at the level of HH ligand to restrict HH signaling during ventral neural patterning. Of note, this mechanism is likely more global, as we also observe defects in somite patterning and craniofacial development.

Despite the overall similarities in patterning defects, there are some clear differences between Mt-Ptch1;Ptch1−/−;Ptch2−/−; Hhip1−/− (total LDA mutant) and Ptch1−/− (complete or near complete LIA mutant) embryos. The floorplate, as demarcated by the highest levels of FOXA2 and SHH synthesis, extends to the dorsal limits of the latter, but only to mid-regions of the neural tube in the former. This may reflect the importance of timing in HH-dependent patterning (Balaskas et al., 2012; Dessaud et al., 2008; Dessaud et al., 2007). In Ptch1−/− embryos, ectopic signaling occurs once cells gain competence to initiate a HH response. Conversely,
the ectopic pathway activity observed in complete LDA mutants is dependent on the kinetics of SHH ligand production and distribution. Dorsal progenitors in LDA mutants ultimately experience high levels of SHH, as indicated by FOXA2 and NKX2.2 expression; however, dorsal cells may not receive this signal within the narrow early competence window required for definitive floorplate specification (Ribes et al., 2010). Last, additional HH-binding proteins may limit the time and range of SHH ligand-based responses, including the HH co-receptors GAS1, CDON and BOC (Allen et al., 2011; Izzi et al., 2011), glypicans (Capurro et al., 2008; Li et al., 2011), megalin (LRP2) (Christ et al., 2012), or other cell-surface proteins.

**Feedback regulation of SHH is required to establish discrete neural progenitor domain boundaries**

At the onset of ventral neural patterning, the SHH gradient induces or represses expression of transcriptional determinants along the DV axis to establish distinct progenitor fates. This initial pattern established by SHH ligand is thought to be inherently disorganized and must be refined by cross-repressive interactions between transcription factors expressed in neighboring domains, resulting in sharp boundaries between neural progenitor populations (Briscoe et al., 1999; Briscoe et al., 2000; Ericson et al., 1997). In fact, mathematical models of the downstream gene regulatory network (GRN) initiated by HH signaling can recapitulate the graded and discrete patterns established in the ventral neural tube independent of threshold responses to HH ligand (Balaskas et al., 2012). This suggests that precise interpretation of a SHH gradient is not required to establish distinct progenitor domains in the ventral neural tube (Balaskas et al., 2012). However, our observation of significant mixing of pV3 and pMN populations in embryos with compromised LDA demonstrates the importance of feedback inhibition at the level of HH ligand to produce sharp boundaries between progenitor populations and suggests that the downstream GRN is not sufficient to properly pattern the ventral neural tube in the context of deregulated HH ligand.

A recent study in zebrafish suggests that the initial noisy pattern established by HH ligand is corrected by dramatic cell rearrangements. These migratory events lead to clustering and positioning of neural progenitors to establish discrete boundaries between domains (Xiong et al., 2013). Strikingly, ectopic motoneurons induced in the zebrafish neural tube migrate into the appropriate region independent of their initial position (Xiong et al., 2013). Conservation of this mechanism in mice would predict that ectopic ventral progenitors in embryos with disrupted LDA should migrate into their appropriate positions and produce discrete boundaries. However, this is not the case, as our analysis reveals significant mixing of ectopic pMN and pV3 cells. This discrepancy suggests that mouse and zebrafish could have fundamentally different mechanisms with which to achieve HH-dependent ventral patterning. Alternatively, the ectopic progenitors in LDA mutants could have been specified after cells are epithelialized and are therefore unable to migrate. One intriguing possibility is that the proper regulation of HH ligands is required to direct these coordinated cell movements, despite an apparent lack of a role for downstream signaling in this process (Xiong et al., 2013).

**Complexity of cell surface regulation of HH signaling during vertebrate embryogenesis**

Together, the data presented here demonstrate that Drosophila and mammals have fundamentally similar feedback responses, though the unique role of Drosophila PTC has been distributed among three partially redundant proteins in mammals (PTCH1, PTCH2 and HHIP1), each of which are direct transcriptional targets of the SHH pathway and each of which participates in direct LDA of SHH signaling (Fig. 7). The presence of additional antagonists may provide essential robustness to HH-dependent patterning processes during vertebrate development, where HH ligands act over significantly larger distances and greater developmental times than during invertebrate embryogenesis and in a broader variety of tissue contexts.

Notably, the collective action of PTCH1, PTCH2 and HHIP1 to restrict HH pathway activity is analogous to the general requirement for the HH co-receptors GAS1, CDON and BOC to activate HH signaling (Allen et al., 2011; Izzi et al., 2011). Similarly, removal of a single co-receptor produces only minor defects in ventral neural patterning while combined removal of GAS1, CDON and BOC reveals their collective requirement in ligand-mediated HH pathway...
activation (Allen et al., 2011). The results presented in this study define an equally important network of cell-surface antagonists that are collectively required to antagonize ligand-dependent HH signaling. However, it remains unclear what characteristics distinguish GAS1, CDON and BOC as HH pathway activators compared with the HH pathway antagonists examined in this study. Future studies will be needed to elucidate the mechanisms that regulate the balance between HH pathway activation and inhibition at the cell surface in different HH-responsive tissues during embryogenesis, organ homeostasis and HH-dependent disease processes.

Acknowledgements
We thank Dr M. P. Scott (Stanford University, CA, USA) for the MT-Patch1 and Patch1 mutant mice, and the Patch1 MEFs. We also acknowledge Dr D. A. Bumcrot (Cunis, Lexington, MA, USA) for the Patch2 mutant mice. We thank Dr Y. Nagakawa (University of Minnesota, Minneapolis, MN, USA) for the DBX1 antibody. The NKX6.1, PAHX, FOXA2, SHH, MNR2, ISL1, VEC1 and EN1 antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. Confocal microscopy was performed in the Microscopy and Image Analysis Laboratory at the University of Michigan.

Funding
Work performed in F.C.’s lab is supported by grants from the Canadian Institute of Health Research (CIHR). F.C. is a Fonds de la recherche en santé du Québec (FRSQ) Senior Scientist. A.M.H. was supported by the University of Michigan MSTP training grant [T32 GM007863] and the National Institutes of Health (NIH) Cellular and Molecular Biology Training Grant [T32GM007315]. J.S. is supported by a research team grant through The University of Michigan Center for Organogenesis. This work was supported by an American Heart Association scientist development grant [11SDG6380000] and by an NIH grant [S2 R21 CA167122-02] to B.L.A. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
Experiments were conceived and designed by A.M.H., A.P.M. and B.L.A., and executed by A.M.H. J.Y.S. and A.M.H. performed the chick electroporations; Executions was performed in the Microscopy and Image Analysis Laboratory at the University of Michigan.

Supplementary material
Supplementary material available at http://dev.biologists.orglookup/ suppl; doi:10.1242/dev.095083

References


