Foxa1 and Foxa2 Are Required for Formation of the Intervertebral Discs

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Abstract

The intervertebral disc (IVD) is composed of 3 main structures, the collagenous annulus fibrosus (AF), which surrounds the gel-like nucleus pulposus (NP), and hyaline cartilage endplates, which are attached to the vertebral bodies. An IVD is located between each vertebral body. Degeneration of the IVD is thought to be a major cause of back pain, a potentially chronic condition for which there exist few effective treatments. The NP forms from the embryonic notochord. Foxa1 and Foxa2, transcription factors in the forkhead box family, are expressed early during notochord development. However, embryonic lethality and the absence of the notochord in Foxa2 null mice have precluded the study of potential roles these genes may play during IVD formation. Using a conditional Foxa2 allele in conjunction with a tamoxifen-inducible Cre allele (ShhcreER²), we removed Foxa2 from the notochord of E7.5 mice null for Foxa1. Foxa1−/−;Foxa2−/−;ShhcreER² double mutant animals had a severely deformed nucleus pulposus, an increase in cell death in the tail, decreased hedgehog signaling, defects in the notochord sheath, and aberrant dorsal-ventral patterning of the neural tube. Embryos lacking only Foxa1 or Foxa2 from the notochord were indistinguishable from control animals, demonstrating a functional redundancy for these genes in IVD formation. In addition, we provide in vivo genetic evidence that Foxa genes are required for activation of Shh in the notochord.

Introduction

Three main structures compose the intervertebral disc (IVD); the annulus fibrosus (AF), the nucleus pulposus (NP), and endplates (EP). A disc is sandwiched between each of the vertebrae along the axial skeleton of vertebrates and allows for flexibility while providing a major support to the body. The NP is a watery gel-like substance containing proteoglycans [1,2]. The NP is formed from the embryonic notochord [3] and is encircled by the AF, a structure comprised of many concentric rings of collagen fibers [1]. The AF is derived from the sclerotome [4], a compartment of the somite. Hyaline cartilage endplates bound the disc superiorly and inferiorly and attach each disc to the vertebrae [1,2]. It is through the endplates that the disc, which is avascular and is not innervated by nerves, obtains its nutrients via diffusion [1,2,5].

During the aging process numerous changes occur to the disc and these are collectively referred to as “disc degeneration” [6–8]. These changes include the transition of the NP from a watery gel-like substance to a more fibrous material, the accumulation of small tears in the AF [2,7,9] and ossification and fracturing of the endplates [10,11]. All of these changes alter the nutrition and structural integrity of the disc, and thus the axial column. In some patients, the NP can herniate out of the AF while in other patients the disc is observed to become compressed resulting in a decrease in the distance between adjacent vertebrae. These changes can result in abnormal pressure being placed on the numerous nerves present in adjacent tissue resulting in severe pain. Back pain, while common, has few effective treatments and is an expensive ailment in terms of medical costs and lost productivity [12,13]. Current treatments address the pain and not the underlying causes, which is frequently disc degeneration. Surgery, and complete or partial disc replacement are options for patients suffering chronic pain but often have high rates of failure and unacceptable side effects for patients [2,9]. It is hoped that by studying the proper formation of the disc and factors that contribute to its degeneration, we can develop novel, effective strategies for disc repair/regeneration.

The notochord is a rod-like structure found in all chordate embryos, and is surrounded by a sheath (the “notochordal” sheath), which is composed of extracellular matrix proteins [14]. During embryonic development the notochord secretes sonic hedgehog (SHH) protein (reviewed in [15]) and serves as a vital signaling center in the chordate embryo [16]. In some species, the notochord also serves as the main structural support for the embryo on which the rest of the body plan is built [16]. The notochord itself forms from the mammalian organizer, or node. This is located at the anterior end of the primitive streak in a mouse embryo.

Cells of the trunk notochord are formed from cells that migrate through the node during gastrulation [17]. The node is capable of inducing a second axis when transplanted into a host embryo [18]. The tail notochord was thought to form from the regression of the notochord sheath, but it is now known that the tail notochord is a derivative of the node itself and is essential for development of the tail and hindgut [19]. Transplantation of mouse notochord into the Xenopus laevis Xenopus laevis embryo results in a secondary axis when the donor notochord is transplanted posteriorly to form the tail notochord [19] [20]. The mouse...
notochord has 3 separate origins; anterior cells that are not node-derived, trunk notochord that forms from cells of the node that undergo convergent extension, and tail notochord which has been proposed to form from cells that have migrated out of the node [19]. The tail bud forms at the end of gastrulation and eventually undergoes secondary neurulation to form the notochord and neural tube in the posterior embryo [21,22].

Foxa genes, formerly termed HNF3 (hepatocyte nuclear factors), were identified by their ability to bind to liver-specific enhancers [23,24]. In mice, the Foxa subfamily has three members, Foxa1, Foxa2 and Foxa3 [25]. They have been extensively studied in several tissues [26-29] and are required for embryonic development and post-natal life [30]. Foxa1 and Foxa2 are expressed in the notochord [25,31-33] and (Figure S1; File S1; Foxa3 is not reported to be found in this structure and was not investigated in the current study.

Foa1 null mice contained a normal notochord and intervertebral discs but died within days of birth as a result of hypoglycemia and dehydration [26]. Foa2 null mice died during early embryogenesis and lacked a node and notochord in addition to containing abnormalities in all germ layers [34,35]. Due to the embryogenesis and lacked a node and notochord in addition to the absence of a node (and thus notochord), potential roles for Foxa genes are functionally redundant in the mouse notochord. Defects in the notochord of double mutants were observed by E9.5, well before the disc starts to form at E12.5. A molecular analysis revealed that Foxa gene expression in the notochord was required for expression of Shh.

Materials and Methods

Animal Care, Generation of Embryos and Genotyping

All mice were raised in accordance with University of Florida Institutional Animal Care and Use Committee Guidelines (UF IACUC) under specific pathogen-free (SPF) conditions. All procedures were approved by the UF IACUC. Animals containing the Foxa1 null allele (Foxa1<sup>−/−</sup>) and Foxa2 conditional allele (Foxa2<sup>ΔN</sup>) were originally generated by the Kaestner laboratory [26,36]. These animals were mated to mice containing the tamoxifen-inducible SihereER<sup>2/2</sup> [38] and R26R [39] alleles to create mice in which Foxa1 or Foxa2 could be deleted from the notochord (Foxa1<sup>ΔN</sup>-Foxa2<sup>ΔN</sup>-SihereER<sup>2/2</sup>-R26R). In this report, these animals are referred to as “double mutants”. Inclusion of the R26R allele allowed for fate mapping of mutant cells. R26R was not present in every animal analyzed. Timed matings were obtained by setting up a cross in the late afternoon; the following morning the female was monitored for the presence of a semen plug, and was by convention determined to have mated at 12:00 am the night before (Day 0.5 when plug is found). Animals homozygous for the Foxa2 conditional allele were phenotypically normal. Weanlings were ear-tagged and genotyped using the tip of the tail. To genotype embryos, extra-embryonic membranes were removed during dissection and lysed in 25 mM NaOH at 95°C for at least 20 minutes. Samples were neutralized with an equal volume of 40 mM TrisHCl and 1–2 μL DNA used in PCR reactions. Genotyping was performed as previously described [26,36,38,39]. At least three mutant animals were examined for each experiment. Control animals were either Foxa1<sup>−/−;Foxa2<sup>−/−</sup> or Foxa1<sup>−/−;Foxa2<sup>ΔN</sup></sup>. Mice were maintained on a mixed genetic background.

RNA in situ hybridization

Anti-sense probes for in situ hybridization were made using a linear template and an in vitro RNA synthesis reaction with Roche reagents. RNA contained a digoxigenin (DIG) label was used. For whole-mount RNA in situ, embryos were dissected in phosphate buffered saline (PBS), fixed overnight in 4% paraformaldehyde (PFA), and then changed to 1% PFA or dehydrated in methanol then bleached with 6% hydrogen peroxide. Embryos were processed for in situ or stored in a −20°C freezer until ready to use. In situ hybridization was performed using standard methods. Briefly, day 1 involved treatment with proteinase K, fixation, and overnight incubation with the antisense probe. On day 2 unbound probe was washed off the sample and antibody against the DIG label was added, day 3 was washes, and on day 4 embryos were developed in BM Purple (Roche) or a mixture of BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) and NBT (nitro blue tetrazolium); both are substrates of alkaline phosphatase, which is conjugated to the anti-DIG antibody. Embryos were developed in the dark; when they were ready they were washed several times with PBS before being fixed and photographed. For section in situ, embryos were dissected in diethyl pyrocarbonate (DEPC)-treated PBS with RNaseZap (Ambion). After overnight fixing in 4% DEPC PFA, embryos were changed to a 30% DEPC sucrose/PBS solution overnight. Finally, embryos were incubated in a 1:1 mix of 30% Sucrose:OCT (Optimal Cutting Temperature, Sakura Tissue-Tek) for two hours, then embedded in 100% OCT and stored at −80°C until use. Embryos were sectioned on a Leica cryostat at 12–14 μm and stored at −80°C. Before proceeding with in situ, slides were dried and sections traced with a hydrophobic pen. All solutions used on Day 1 were DEPC treated and 10% dextran sulphate was added to the prehybe solution. Otherwise procedure was similar to whole-mount in situ hybridization. Slides were coverslipped with paraffin for overnight incubations. The protocol was based on previously published in situ protocols [40,41] and resources from the Tabin lab website (Harvard University). An unabridged in situ protocol is in the File S1. Probes used have been described previously: Shh [42], T [Bachury] [43], Noto [44], Tbx18 [45], Pax1 [46], Pax3 [47], Nkx2.2 [48], Nkx6.1 [48], and Pch1 [49]. Foxa1 and Foxa2 probes were made from IMAGE clones from Open Biosystems: Foxa1 (4911145) and Foxa2 (6488102).

LacZ staining

Embryos were collected and fixed in 0.2% PFA overnight. The next day embryos positive for the R26R and SihereER<sup>2/2</sup> alleles were rinsed 3×10 minutes in LacZ rinse buffer (0.1M sodium phosphate [pH 7.4], 0.1% sodium deoxocholate, 2 mM MgCl2, 0.2% NP-40) before being put in LacZ staining solution (1 mg/mL X-gal in DMF, 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6) overnight in the dark at room temperature. Reactions were stopped the following day by 3×10 minute washes in PBS. Embryos were photographed and then fixed overnight in 4% PFA.

Histology

Fixed tissue was dehydrated in an ethanol series before being embedded in paraffin by standard methods and sectioned at 7 μm on a microtome. LacZ-stained tissue was sectioned at 10 μm. Slides were dewaxed in xylenes for 10 minutes followed by
rehydration in an ethanol series for 10 minutes each. Slides were washed for 15 minutes in Alcian blue stain (Sigma, pH 2.5), followed by 10 minutes in tap water. Picrosirius red (PolyScientific) staining was done for 45 minutes and then an acid wash followed (0.025% acetic acid in deionized water), followed by de-staining for 1 minute each in 95% and 100% ethanol, then 10 minutes in xylene. Slides were mounted with Permount (Fisher Scientific) and coverslipped. For LacZ-stained slides, they were dewaxed and rehydrated as described and then counterstained with Nuclear Fast Red for 5 minutes, and then de-stained, xylene washed and coverslipped as above.

**Nuclear Fast Red & Alcian blue staining**

Embryos embedded in OCT were serially sectioned at 10 μm on a cryostat. Slides was washed in Alcian blue for 15 minutes, followed by 10 minutes in tap water, 5 minutes in Nuclear Fast Red, and then dipped in deionized water before being mounted with Glycergel (DAKO).

**Immunohistochemistry**

Tissue was fixed in 0.2% PFA overnight and embedded in paraffin. Paraffin slides were dewaxed and rehydrated as described above, and then antigen retrieval was performed in a conventional microwave at 70% power for 15 minutes in 10 mM sodium citrate buffer +0.1% Tween 20. Slides were allowed to cool for 30 minutes and then were blocked for 30 min to 2 hours with 10% donkey serum in PBST. Primary antibody for FOXA2 (AbCam, ab5074) was added at 1:75 and LAMININ (DAKO, Z0097) 1:200 in 10% donkey serum, coverslipped with paraffin, and left overnight at 4°C. The next day slides were washed with PBST several times, blocked again for 30 minutes in 10% donkey serum/PBST, and secondary antibody was added at 1:300 for 1 hour (AlexaFluor 568 donkey anti-goat IgG, Invitrogen and DyLight 488 donkey anti-rabbit IgG, Jackson Immunoresearch). After washing with PBST and counterstaining with DAPI, slides were mounted with DAKO Fluorescent mounting media and coverslipped.

**LysoTracker assay**

Embryos were dissected into PBS that had been warmed to 37°C. LysoTracker Red reagent (Invitrogen) was added (25 μL to 5 mL warm PBS) and 500 μL was added to each embryo. Embryos were incubated at 37°C in the dark for 30 minutes and then rinsed several times with PBS before being fixed in 4% PFA overnight. The next day, embryos were dehydrated in a methanol series for at least 30 minutes each in 25%, 50%, 75%, and 100% methanol. Embryos were stored at -20°C in the dark until photographed.

**Results**

**Removal of Foxa1 and Foxa2 in the mouse notochord**

The Foxa1 null allele and the Foxa2 conditional floxed allele have been described previously [26,36]. Mice containing these alleles were crossed to animals containing the ShhcreERT2 allele [38] to generate Foxa1+/−;Foxa2+/−;ShhcreERT2 mice. These animals were phenotypically normal since the ShhcreERT2 allele only produces CRE protein in the presence of tamoxifen. Double mutant embryos were obtained by crossing Foxa1+/−;Foxa2+/−;ShhcreERT2 males to Foxa1+/−;Foxa2−/− females (diagrammed in Figure 1A). Tamoxifen was administered at E7.5 by oral gavage to remove the Foxa2 conditional allele in embryos that were homozygous for the Foxa1 null allele. In this report, mice that were null for Foxa1 and had Foxa2 removed in Shh-expressing cells are referred to as “double mutants”.

**Figure 1. Strategy to remove FOXA2 from the mouse notochord.** A: Mice heterozygous for the Foxa1 null allele (Foxa1+/−), homozygous for the Foxa2 floxed conditional allele (noted as Foxa2−/− in this report) and that contained the ShhcreERT2 allele were crossed to Foxa1+/−;Foxa2−/− mice to create double knockouts. The pregnant dam was given tamoxifen by oral gavage (4 mg) in corn oil when embryos were E7.5. * indicates that the R26R allele was crossed into some animals for fate-mapping. B: Exposure of E7.5 embryos to tamoxifen that contained the ShhcreERT2 and CRE-inducible R26R LacZ reporter resulted in reporter expression in the notochord (NC) but not the floorplate. The ShhcreERT2 allele is not active in the floor plate at E7.5. Scale bar is 100 μm. The ventral neural tube is outlined in black for clarity. C–F: Confirmation of removal of Foxa2−/− from the notochord using a FOXA2-specific antibody. C and D: FOXA2 protein was present in the notochord and floorplate of control embryos (C) but absent from the notochord of Foxa2−/−;ShhcreERT2 embryos (D). The notochord is outlined in white. E and F: The presence of a notchord in Foxa2−/−;ShhcreERT2 embryos was confirmed by staining for Laminin protein. Dissociation of the notochord from the neural tube in D and F is likely a result of tissue processing. G and H: DAPI staining of a similar E9.5 section to show nuclear staining. White arrows in C–H denote the notochord. C–H: Scale bar is 50 μm.

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had been given a single dose of tamoxifen at E7.5. These data are consistent with our previous published report in which we demonstrated that tamoxifen administration at E7.5 drives CRE expression in the notochord but not the floor plate [50].

To confirm the removal of Foxa2 from the notochord in tamoxifen-treated embryos, immunohistochemistry for FOXA2 was performed using an antibody raised to a part of the protein that is deleted upon CRE-induced recombination [31]. FOXA2 protein was found in the floorplate and notochord of E9.5 control embryos that had been treated with tamoxifen at E7.5 but lacked the ShhcreERT2 allele (Figure 1C). In Foxa2+/−; ShhcreERT2 embryos, FOXA2 was present in the floorplate but was absent from the notochord (Figure 1D). Immunohistochemistry was performed for Lammin protein, which among other structures, outlined the notochord demonstrating that despite a lack of FOXA2 staining in the notochord (Figure 1D). Immunohistochemistry was performed for each other during formation of the intervertebral discs and vertebral columns of controls and mutants were analyzed using Alcian blue and picrosirius red stains. Picrosirius red stains collagens [52,53] while Alcian blue stains cartilage and mucopolysaccharides [54].

Control animals had fully-formed intervertebral discs with clearly identifiable nuclei pulposi and annuli fibrosi (Figure 2A,E and Figure 2A,F). It has been reported that expression of CRE protein can cause phenotypes in some tissue types [55]. To determine if CRE protein produced from the ShhcreERT2 locus could cause disc defects, Foxa2+/−; ShhcreERT2 animals were examined in detail. An extensive histological analysis revealed that the vertebral columns from these embryos were indistinguishable from controls (Figure 2C,G and Figure 2C,G). These data also indicated that removal of only Foxa2 from the notochord was not sufficient to cause a phenotype in the vertebral column. In addition, removal of only Foxa1 from all cells (Foxa1−/−;Foxa2−/− embryos) also resulted in normal NP and vertebrae (Figure 2B,F and Figure 2B,F). For more details, see Supporting Information.

To determine if the lack of a phenotype in individual Foxa mutants was a result of compensation by the remaining gene, both Foxa1 and Foxa2 were removed from the embryonic notochord (Foxa1−/−; Foxa2−/−; ShhcreERT2). In double mutants the NP was compressed and small (Figure 2D and H). The disc defects were generally more severe posteriorly than anteriorly. Double mutant embryos, had shorter tails (Figure 2F), that frequently lacked disc and vertebral structures (Figure 2H). The ossification centers were also abnormal in the double mutants (Figure 2D) in comparison to control (Figure 2A) and single knockouts (Figure 2A,B). These data suggest that Foxa1 and Foxa2 can compensate for each other during formation of the intervertebral discs and vertebrae, consistent with reports in other tissues in which phenotypes were only observed when both genes were removed from the same tissue [27,29].

**The notochord to nuclei pulposi transition is abnormal in Foxa1;Foxa2 double mutants**

Nuclei pulposi form entirely from the embryonic notochord [3]. To determine if the notochord to nuclei pulposi transition was abnormal in Foxa1;Foxa2 double mutants, notochord cells were fate mapped using the R26R CRE-inducible reporter allele [39]. The R26R reporter allele expresses LacZ in all cells in which CRE protein has been expressed. Embryos containing the ShhcreERT2 allele that have been exposed to tamoxifen at E7.5 express LacZ in the notochord. Importantly, once cells express LacZ they, and all their descendants will express LacZ even if CRE protein is not longer present.

Double mutants containing the R26 allele were harvested at E17.5 and treated with β-galactosidase to determine the fate of notochord cells. In control embryos (Figure 2L) and embryos lacking Foxa2 (Figure 2J,M) notochord cells formed nuclei pulposi. In contrast, in double mutant embryos notochord cells were observed to reside throughout the vertebral column (Figure 2K,N). It was not possible to follow the fate of notochord cells in animals from the floxed Foxa2 allele since inclusion of the Cre allele would have resulted in deletion of the floxed Foxa2 allele, thus we used mice containing the Shhfluc allele which constitutively express Cre in all Shh descendants including the dorsal root ganglia (Figure 2I, [3]). It was also not possible to obtain a Foxa1 null sample for LacZ staining due to the construction of our mating scheme (see Figure 1A).

**Cell death occurs in posterior somites and the tail of Foxa1;Foxa2 double mutants**

To examine cell death in Foxa1;Foxa2 knockout embryos, LysoTracker Red reagent (Invitrogen) was used. At E10.5 similar levels of cell death were observed in control, single and double mutants (Figure 3A–D). However, at E11.5 Foxa1;Foxa2 double mutants displayed massive cell death in the posterior somites and midline of the tail (Figure 3H,L) compared to control (Figure 3E,I), Foxa1 null (Figure 3F,J), and Foxa2 notochord mutant (Figure 3G,K) littermates. This dramatic amount of cell death likely results in the shorter tail found in E17.5 (Figure 5D) and E19.5 double mutants. Due to the presence of ectopic cell death in double mutants beginning at E11.5, all gene expression analysis was performed prior to this embryonic stage.

**Foxa expression in the notochord is not required for formation of the sclerotome**

Pax1 and Pax9, two transcription factors expressed in the sclerotome, are involved in the development of the vertebral column. Pax1;Pax9 double mutant mice do not form IVs and lose the segmental arrangement of vertebrae [56]. In this study we examined Pax1, which is expressed in the early sclerotome, using section RNA in situ hybridization. Pax1 was expressed robustly in the sclerotome in the E10.5 forelimb (Figure S3A-D and File S1) and hindlimb levels of all embryos (Figure 4A-D). Tbx18 maintains the anterior-posterior polarity of the somites, and its loss results in abnormalities of the axial skeleton [57]. Tbx18, expressed in the anterior somite [57,58], was also found to be indistinguishable from control (Figure 4E), single knockout (Figure 4F and G), and Foxa1;Foxa2 double mutants (Figure 4H) at E10.5.

**Noto and T (Brachyury) expression in Foxa1;Foxa2 double mutants**

The transcription factors Noto and T (Brachyury) were examined by whole-mount RNA in situ hybridization. Noto is responsible for the truncate (t) mutation in mice, which affects development of the posterior notochord resulting in the lack of a tail [44]. The zebrafish homologue of Noto, fjh (Boating head), results in the absence of the notochord [59]. Noto expression is reported to be
dependent on the initial expression of both Foxa2 and T [44]. To
determine if Noto was downstream of Foxa, RNA in situ
hybridization was performed on E9.5 and E10.5 double mutant
embryos. At this stage of development, FOXA2 protein is absent
in the notochord of mutant embryos (see Figure 1D). In E9.5
double mutants and littermates, Noto was robustly expressed
throughout the tail (Figure 5A–D). By E10.5 Noto was not
detectable in the tail of double mutants (Figure 5H) but was
found in the tails of control (Figure 5E), Foxa1 null (Figure 5F), and
Foxa2 notochord knockouts (Figure 5G).

T is expressed in the notochord [60,61] and mice heterozygous
for a mutation in this gene have been reported to have shorter tails
[62]. In Foxa2 null embryos the notochord does not form and as a
result T is not expressed. In controls (Figure 5I), mice that lacked
Foxa2 in the notochord (Figure 5K), Foxa1 null embryos (Figure 5J),
and double mutants (Figure 5L), T was expressed throughout the
posterior notochord at E10.5. The massive cell death observed in
E11.5 embryos precluded an analysis of T expression at later
stages.

The notochord sheath is abnormal in double mutants

The notochord sheath, composed of an acellular extracellular
matrix, surrounds the notochord the length of the embryo. To
examine the notochord sheath, transverse sections were stained
with alcian blue [14] and nuclear fast red was used as a
counterstain. An in situ hybridization for T (Brachyury) was also
performed on adjacent sections in order to accurately visualize the
notochord (insets in Figure 5M–P). In control embryos at the
forelimb level (Figure 5M), the notochord sheath was observed to
surround the notochord. Single knockout embryos also contained
a readily apparent notochord sheath (Figure 5N, O). In contrast,
double mutant embryos, which did have a notochord visible at the
forelimb level (Figure 5P), contained a much more faint notochord
sheath.

Expression of Shh and activation of the hedgehog
signaling pathway requires Foxa gene expression in the
notochord

Shh is expressed in the notochord and is required for the
formation of IVDs [42,50,63,64]. Shh and hedgehog signaling have
also recently been demonstrated to be required for post-natal

Figure 2. Foxa1;Foxa2 double mutants have severe defects in intervertebral disc formation. A–H: Alcian blue and picrosirius red staining
of disc sections. Scale bar is 200 μm. Thoracic sections at E19.5 show a normal sized nucleus pulposus (NP) surrounded by a red-stained annulus fibrosus (AF) in control (A) Foxa1 null (B) and Foxa2 notochord knockout (C) embryos. Asterisk in Foxa1−/−;Foxa2−/− column denotes that embryo was E17.5. Disc morphology was also normal at the lumbar region in these mice (E, F, G). In Foxa1;Foxa2 double mutants (D and H) nuclei pulposi were abnormal. Nuclei pulposi were observed to be much smaller than nuclei pulposi present in control or individual single mutants. I and J: A fatemap of notochord cells using the R26R reporter allele demonstrated proper nuclei pulposi formation in control (I) and Foxa2 notochord knockouts (J). In contrast, Foxa1;Foxa2 double mutants (K) had severely deformed nuclei pulposi and blue cells were dispersed. Defects were more severe in the posterior. (I) is an embryo of the genotype ShhgfpcreR26R. White asterisk marks dorsal root ganglia. The Shhgfpcre allele is constitutively active, therefore all Shh-expressing cells, including dorsal root ganglia, and their descendants are LacZ-positive. All LacZ positive cells in J and K are derived exclusively from the notochord [3]. In our mating it was not possible to obtain a Foxa1−/− notochord fatemap without also removing the floxed Foxa2 allele, therefore the Foxa1−/− fatemap is not shown (N/A = not applicable). L, M and N: Sections of LacZ-stained tissue at the lumbar level. Inset in N shows an additional section outside of the plane of the NP; numerous cells are scattered throughout the disc region and vertebral bodies. L–M: Scale bar is 100 μM. Arrow in L denotes a notochord remnant, which is a notochord cells that does not reside in the NP.

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growth and differentiation of the mouse disc [65]. The Shh notochord enhancer element has been identified and shown to contain binding sites for FOXA family members [66] suggesting that FOXA proteins directly regulate expression of Shh within the notochord. The apparent functional redundancy between FOXA family members and the early lethality observed in Foxa2 null embryos has precluded a genetic analysis of the role FOXA proteins may play in activation of Shh and the hedgehog signaling pathway in the vertebrae notochord.

To analyze the role FOXA proteins may play in regulating Shh, E10.5 double mutant embryos were initially analyzed using RNA in situ hybridization. In control (Figure 6E,I), Foxa1 null (Figure 6F,J) and Foxa2 notochord knockout (Figure 6G,K) embryos had numerous LysoTracker positive cells in the somites, dorsal tail, and end of the tail but were indistinguishable from one another. In contrast, double mutant embryos (H and L) had numerous LysoTracker positive cells in the somites and midline of the tail (white arrows). Images I–L are zoomed in areas denoted by the boxes in E–H. The insets in E–H are brightfield images of E11.5 embryos. The scale bar for the LysoTracker images in E–H is 1 mm.

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Figure 3. Cell Death is increased in the tail of E11.5 but not E10.5 Foxa1;Foxa2 double mutant embryos. Cell death was assayed using LysoTracker on live embryos at E10.5 and E11.5. A–D. Control (A), Foxa1 null (B), Foxa2 notochord knockouts (C), and Foxa1;Foxa2 double knockout embryos (D) had similar amounts of cell death at E10.5. Inset is a brightfield image of an E10.5 embryo shown for reference. Scale bar for the LysoTracker is 1 mm. E–H: At E11.5, control (E and I), Foxa1 null (F, J) and Foxa2 notochord knockout (G and K) embryos had numerous LysoTracker positive cells in the somites, dorsal tail, and end of the tail but were indistinguishable from one another. In contrast, double mutant embryos (H and L) had numerous LysoTracker positive cells in the somites and midline of the tail (white arrows). Images I–L are zoomed in areas denoted by the boxes in E–H. The insets in E–H are brightfield images of E11.5 embryos. The scale bar for the LysoTracker images in E–H is 1 mm.

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Figure 4. The sclerotome is initially unaffected in Foxa1;Foxa2 double knockouts. A–D: Pax1 expression at the hindlimb level (transverse sections are shown). Pax1 is expressed in the sclerotome at E10.5. In controls (A), Foxa1 nulls (B), Foxa2 notochord knockouts (C), and double mutants (D) there was no difference in Pax1 expression. E–H: E10.5 sagittal sections showing Tbx18 expression at mid-trunk level. Tbx18 is confined to the anterior sclerotome. No differences in expression of Tbx18 were observed in single (F and G) and double (H) mutants compared to control embryos (E). Scale bars are 200 μm.

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decreased in the notochord of the tail and absent from the floorplate (Figure 6H and L). Shh was expressed robustly in the posterior limbs of double mutants (Figure 6H), consistent with the absence of CRE expression in these tissues (at E7.5, the time point of tamoxifen exposure, the ShhcreERT2 allele is not expressed in the limbs since these structures have not yet formed). To examine if this decrease of Shh occurred earlier, we also performed in situ hybridization with E9.5 mice. Control (Figure 6A), Foxa1 null (Figure 6B), and Foxa2 notochord knockout (Figure 6C) mice had robust expression of Shh in the brain, hindgut, and midline. In contrast, the double mutant embryos lost Shh from the midline in the distal half of the body, though it was maintained in the hindgut and brain (Figure 5C, arrow).

To confirm a decrease in hedgehog signaling in the notochord of double mutants, Pcth1, a direct target of the hedgehog pathway, was examined [49,67]. In the tails of double knockout embryos, Pcth1 was decreased (Figure 6P) compared to control littermates and embryos that had either Foxa1 or Foxa2 absent in the notochord (Figure 6M,N, and O).

**Foxa expression in the notochord is required for dorsal-ventral patterning of the neural tube**

The notochord is an important embryonic signaling center that is required for patterning the floorplate [16]. Shh secreted from the notochord patterns the floorplate in a concentration-dependent manner [68]. Since Shh was decreased in Foxa1;Foxa2 double mutants (Figure 6), it was possible that neural tube patterning would be aberrant. Furthermore, dorsal ventral mispattering of the neural tube has been described in Foxa2 null mice [34,35]. Nkx6.1, a transcription factor that is typically expressed in the ventral half of the neural tube [48], was found to be normal at the hindlimb level of control [16]. Nkx6.1 expression was undetectable at the hindlimb level in double mutants (Figure 7H). Expression was present in the ventral neural tube of controls and single mutants (Figure 7E, F and G). Pax3, which is expressed in the dermomyotome and dorsal neural tube [47], was examined in E10.5 embryos (Figure 7I–L). At the hindlimb level, Pax3 was
extended ventrally in the neural tube of Foxa1;Foxa2 double mutants (Figure 7L). Dermomyotome expression of Pax3 appeared unchanged in double mutants. At the level of the double mutant forelimb, expression of all genes examined was indistinguishable from controls (Figure S3 and File S1).

**Discussion**

*Foxa1* and *Foxa2* are functionally redundant in the notochord

*Foxa2* null mice die lacking the node, which during later embryonic development forms the notochord [34,35]. To bypass the early requirement for Foxa2 in node formation, we removed Foxa2 from the notochord using a tissue-specific Cre line. In these mice, Foxa2 was expressed in the node but not the notochord, which allowed for the characterization of the role this gene played in the notochord and disc development. In Foxa1;Foxa2 double mutants, Foxa1 was absent from all cells (null allele) while Foxa2 was only removed from the notochord in the vertebral column. Since Foxa1 null mice die postnatally and have fully-formed discs ([26] and this report) the phenotypes present in the double mutants were likely due to the removal of both of these genes from the notochord. However, we cannot rule out the possibility that Foxa2 expression in the floorplate plays a role in disc development in vertebral columns that lack Foxa2 in the notochord. In our study, Foxa2 was not removed from the floorplate since the Shh promoter was not active in the floorplate during the time that embryos were exposed to tamoxifen.

The caudal vertebral column is more severely affected in Foxa1;Foxa2 double knockouts

A common feature present in most mouse mutants that have abnormal notochord development is that the caudal part of the animal is more severely malformed than the rostral part [44,61,63]. Consistent with these reports, the tails of double mutant Foxa1;Foxa2 mice lacked any discernable vertebrae or disc structures at birth. The only exception was two animals that had shortened tails with discs in them, but thoraco-lumbar discs were compressed and small. It is possible that in these animals that
tamoxifen was administration at slightly later stages since plug dates are only estimated. In double mutant animals, defects were less severe in the rostral region of the embryo with well-developed discs found above the forelimb level in double mutants.

The forming notochord has been fate mapped and found to be composed of three distinct regions: head notochord which is not derived from node cells, trunk notochord that is node-derived, and tail notochord that forms from migrating node cells [19]. The increasing severity of notochord defects in the caudal region of the embryo may be due to the notochord being derived from different regions of the embryo. The ShhcreERT2 allele used in the current study to remove Foxa2 is expressed throughout the notochord [3]. However, it is currently unknown if this Cre allele is expressed in all regions of the notochord at E7.5, the time that tamoxifen was administered to remove Foxa2. It is possible that CRE protein driven from the ShhcreERT2 allele is first expressed in caudal notochord cells, which would result in a transient burst of FOXA2 protein expression in the anterior notochord prior to CRE-induced recombination of the Foxa2 allele.

Despite the fact that there is no tail or posterior notochord at E7.5, when we administer tamoxifen, defects were observed in the tail. This phenotype could be due to knockout of Foxa2 (and lack of Foxa1) in the cells of the node/notochord that are normally supposed to produce this tissue. Foxa2 will be active for a short period of time before tamoxifen can inactivate this gene. It is possible that a short burst of Foxa2 transcription is sufficient to begin the formation of the posterior notochord, but the subsequent failure of hedgehog signaling in these cells may prevent the rest of the notochord from forming normally.

**Figure 7. Dorsal-ventral patterning of the neural tube is abnormal in Foxa1;Foxa2 double knockouts.** Section in situ hybridization at E10.5 for Nkx6.1 (A–D). Control (A), Foxa1 nulls (B) and Foxa2 notochord knockout (C) embryos had similar expression patterns in the ventral neural tube. However, in double Foxa1;Foxa2 knockouts (D), Nkx6.1 mRNA was restricted to a ‘wedge’ shape at the bottom of the neural tube. E–H: Nkx2.2 was constricted to a ventrally-located band of cells in the neural tube in controls (E), Foxa1 nulls (F) and Foxa2 notochord knockout (G) embryos. In Foxa1;Foxa2 double mutants, Nkx2.2 was undetectable (H). I–L: Pax3 is normally expressed in the dorsal neural tube and in the dermomyotome. In control (I), Foxa1 nulls (J) and Foxa2 notochord knockouts (K) Pax3 was expressed normally. In double mutants (L), Pax3 expression was expanded ventrally (black bar). Pax3 expression in the dermomyotome of double mutants was normal (L). A–H: Scale bar 100 μm, I–L: Scale bar 200 μm. doi:10.1371/journal.pone.0055528.g007

**Foxa1 and Foxa2 are required for activation of the hedgehog signaling pathway in the notochord**

SHH protein secreted from the notochord is required to pattern the neural tube, for formation of the notochordal sheath that surrounds the notochord and formation of nuclei pulposi [15,42,50,63,64]. In post-natal mice, SHH from the NP is also required for disc differentiation and growth [65]. In Foxa1;Foxa2 double knockouts, Shh mRNA was undetectable in the tail by E9.5 and decreased in the notochord at the hindlimb level of E10.5 embryos. Foxa2 has been proposed to activate transcription of Shh in the notochord [34,35] and other embryonic tissues [29,37] suggesting that FOXA proteins directly regulate the transcription of Shh in the embryonic notochord.

A previous report identified a Shh notochord enhancer that was sufficient to drive reporter expression specifically in the notochord [66]. This enhancer element contained three Foxa binding sites that were required for reporter gene expression in the notochord [66]. However, due to the early lethality of Foxa2 null embryos and functional redundancy between FOXA proteins, previous studies have been unable to confirm Shh regulation by FOXA proteins in vivo. Our data directly demonstrate that expression of Shh in the notochord requires FOXA proteins, suggesting that the previously identified FOXA binding sites in the Shh notochord enhancer are functional. Consistent with these data Pch1 expression, a direct target of hedgehog signaling, in the caudal region of Foxa1;Foxa2 double knockouts was also reduced.

The observed defects in the notochord sheath in double mutants are also consistent with a decrease in hedgehog signaling [63]. The lack of a sheath to contain cells within the notochord in double mutants may cause the observed dispersal of LacZ-stained notochord cells in our fate-mapping experiment. Without the
notochordal sheath to maintain cells within the notochord; these cells may be pushed to ectopic locations during formation of the vertebral column.

The phenotype of the Foxa1;Foxa2 double mutant mouse described in this report is similar to mice that we previously characterized in which hedgehog signaling was removed from both the notochord and floorplate [63]. In our previous report, Smoothened was removed and disc development assessed. The NP was found to be small and compressed, and the notochord to nucleus pulposus transition was also abnormal. Choi et al did not observe the dorso-ventral mispatterning of the neural tube observed in Foxa1;Foxa2 double mutants, possibly because Shh is still being expressed from the notochord in mice that lacked Smoothened from the notochord. We have also previously shown that removal of Shh from the floorplate had no affect on disc and vertebral development, while removal of Shh from the notochord resulted in a severe deformation of the entire vertebral column and decreased Pax1 expression at E11.5 [30].

The neural tube was “dorsalized” in Foxa1;Foxa2 double mutants

Studies using explant cultures have shown that Shh signaling counteracts expression of genes that are required for formation of the dorsal neural tube [69]. The expansion of dorsally expressed genes in the neural tube and the loss of ventral neural tube identity and motor neurons have been documented in the Foxa2 null mouse [34,35]. A similar phenotype has also been reported in the Shh null mouse [64]. In mice in which Foxa2 was absent in all cells (null animals), Fox3 was expanded throughout the entire neural tube [34] while markers of motor neurons such as Islet-1 were reported to be absent from the neural tube [33].

In double Foxa1;Foxa2 mutants, in which Foxa2 was absent from the notochord, expression domains of dorsal neural tube genes were expanded at the expense of ventrally expressed genes (a “dorsalized” neural tube). The loss of dorsal-ventral identity in the neural tube of double Foxa1;Foxa2 mutants was consistent with the observed decrease in Shh expression in the notochord.

In the caudal region of double Foxa1;Foxa2 mutants, the floorplate appeared to be absent. The Foxa2 null mouse has also been reported to lack a floorplate (presumably because no notochordal forms in null embryos resulting in the absence of notochord-produced SHH protein) [34,35]. We speculate that the floorplate in Foxa1;Foxa2 double mutants does not form in the caudal embryo due to a requirement of FOXA expression in the notochord, which is required to activate Shh [68].

Noto expression is not maintained in Foxa1;Foxa2 double knockout embryos

Noto has been proposed to be a target of FOXA2 since it is not expressed in chimeras between Foxa2 null ES cells and wild-type tetraploid embryos [44]. Tetraploid chimeras between Foxa2 null ES cells and wild-type embryos have been described, and while they do not form the node or notochord, primitive streak morphogenesis is rescued [70]. In our experiments, Noto was robustly expressed in the notochord and node of all double mutant embryos examined at E9.5. However at E10.5, Noto was absent from the tail of double knockout embryos. The presence of a notochord in E10.5 embryos was confirmed by analysis of T, which is expressed in the caudal notochord. These data suggest that Noto requires FOXA in the notochord to maintain its expression. However, it is currently unclear if FOXA proteins directly regulate Noto expression or if transcription of this gene is downstream of the hedgehog signaling, or a yet unidentified signaling pathway.

It is likely that other pathways besides hedgehog signaling are responsible for disc formation. Removal of hedgehog signaling from the post-natal disc both in vivo and in vitro was reported to decrease TGF-β and Wnt signaling [65]. In addition, Wnt signaling was shown to be required for posterior tail development. Early in development though, Foxa1 and Foxa2 are both still expressed in the β-catenin knockout notochord [20]. Finally, a recent screen of Foxa2 targets in the notochord identified seven genes that were previously not recognized as Foxa2 targets expressed in the node, posterior notochord, and throughout the entire notochord [71]. It is possible that one or more of these genes may be downregulated in Foxa1;Foxa2 double knockout.

Our data demonstrates that both Foxa1 and Foxa2 are required for disc formation. The loss of both Foxa1 and Foxa2 from notochord cells results in an aberrant notochord to NP transition, cell death in the posterior somites and tail, and severe deformation of the NP. Our data provide in vivo evidence for Shh being a direct downstream target of the Foxa signaling pathway in the notochord suggesting that the previously identified Foxa binding sites in the Shh notochord enhancer are functional.

Supporting Information

Figure S1 Foxa1 and Foxa2 expression in the notochord. A–D: Whole-mount Foxa1 in situ hybridization. I–K: Section in situ hybridization for Foxa1. Foxa1 is undetectable at E7.5 (A), but expressed in the notochord at E8.5 (B–D,I,J), floorplate (B–D,J), midbrain and gut (B–D, I). It remains on in the notochord until E12.5 (not shown). Foxa1 is not expressed in the NP at E14.5 (K). E–H: Whole-mount Foxa2 in situ hybridization. L–N: Section in situ hybridization for Foxa2. Foxa2 is detectable at E7.5 (E) and remains faintly in the notochord until E8.5 (F,L). It is also expressed in the floorplate, gut, and midbrain (F–H). Foxa2 remains detectable in the floorplate until at least E14.5 (F–H, L–M, and data not shown). Purple staining in brain vesicles in (G) is signal pooling in the brain. Scale bars are 50 μm for I and L, 100 μm for J and M, and 200 μm for K and N. Black arrowhead in L,J,L and M point to the notochord. (TIF)

Figure S2 Vertebral abnormalities and shortened tail in double mutant. A–D: Picrosirius red and Alcian blue staining of E19.5 control (E), Foxa1 null (F), Foxa2 knockout (G), and double Foxa1;Foxa2 knockout embryos (H). I–L: Nkx2.2 at the
forelimb level. Mxα2.2 is expressed just dorsal to the floorplate in control (I), Foxa1 null (J), Foxa2 notochord knockout (K), and double Foxa1;Foxa2 knockouts (L). M–P. Pax3 at forelimb level. Pax3 is expressed in the dermomyotome and dorsal half of the neural tube in control (M), Foxa1 null (N), Foxa2 notochord knockout (O), and double Foxa1;Foxa2 knockouts (P). Scale bars: A–D, M–P: 200 μm. E–L: 100 μm.

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Author Contributions

Conceived and designed the experiments: JM BDH. Performed the experiments: JM YL. Analyzed the data: JM YL BDH. Contributed reagents/materials/analysis tools: BDH. Wrote the paper: JM BDH.

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