RESEARCH ARTICLE

A developmental transcriptomic analysis of Pax1 and Pax9 in embryonic intervertebral disc development

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ABSTRACT

Pax1 and Pax9 play redundant, synergistic functions in the patterning and differentiation of the sclerotomal cells that give rise to the vertebral bodies and intervertebral discs (IVD) of the axial skeleton. They are conserved in mice and humans, whereby mutation/deficiency of human PAX1/PAX9 has been associated with kyphoscoliosis. By combining cell-type-specific transcriptome and ChIP-sequencing data, we identified the roles of Pax1/Pax9 in cell proliferation, cartilage development and collagen fibrillogenesis, which are vital in early IVD morphogenesis. Pax1 is up-regulated in the absence of Pax9, while Pax9 is unaffected by the loss of Pax1/Pax9. We identified the targets compensated by a single- or double-copy of Pax9. They positively regulate many of the cartilage genes known to be regulated by Sox5/Sox6/Sox9 and are connected to Sox5/Sox6 by a negative feedback loop. Pax1/Pax9 are intertwined with BMP and TGF-β pathways and we propose they initiate expression of chondrogenic genes during early IVD differentiation and subsequently become restricted to the outer annulus by the negative feedback mechanism. Our findings highlight how early IVD development is regulated spatio-temporally and have implications for understanding kyphoscoliosis.

KEY WORDS: Pax1, Pax9, Sox trio, Intervertebral disc, TGF-B, BMP

INTRODUCTION

The vertebral column is the fundamental infrastructure of the vertebrate body, composed of vertebral bodies (VBs) linked together by fibro-cartilaginous intervertebral discs (IVDs). During mouse embryogenesis, the vertebral column (VC) is formed from the somites through a series of precisely regulated processes. The ventro-medial cells of the somite become specified to a sclerotomal fate by Sonic hedgehog (Shh) signals derived from the notochord, which also require the maintenance of a bone morphogenetic protein (BMP)-reduced zone by BMP antagonists (Fan and Tessier-Lavigne, 1994). Shh induces the expression of Paired-box 1 (Pax1), Paired-box 9 (Pax9) and Mesenchyme forkhead-I (Mfh1) in the ventral somites which convey their proliferative function (Furumoto et al., 1999). The sclerotomal cells then migrate to surround the notochord and condense to form the mesenchymal prevertebrae, which is further segmented into condensed and less condensed regions along the anterior-posterior axis of the embryo, at around embryonic day (E) 12.5. The former gives rise to IVD anlagen mesenchyme while the latter develop into VBs that subsequently undergo endochondral ossification. The IVD mesenchyme then differentiates into cartilaginous inner annulus (IAF) and a fibrous outer annulus (OAF) (Sivakamasundari and Lufkin, 2012; Smith et al., 2011).

Pax1 and Pax9 which belong to the same subfamily of Pax genes, encode transcription factors (TFs) that contain a highly conserved DNA-binding domain, the paired box (Paixao-Cortes et al., 2015). They are co-expressed in, and are critical for, the development of sclerotome-derived VBs and IVD anlagen (Neubüser et al., 1995). While both Pax1 and Pax9 are expressed uniformly in the condensing cells of the IVD anlagen at E12.5, with further differentiation their expression declines within the IAF and becomes restricted to the OAF by E15.5 (Wallin et al., 1994). Studies on the Pax1 undulated mutants and the targeted Pax1−/− and Pax9−/− knock-out (KO) mutants revealed their redundant roles in axial skeletogenesis – Pax1 was able to fully compensate for the loss of Pax9, while Pax9 was inadequate to promote normal development of the VC in the absence of Pax1 (Wallin et al., 1994; Wilm et al., 1998). An analysis of the Pax1/Pax9 multiple allele KO mutants uncovered their synergistic roles in axial skeleton development, demonstrating a clear gene-dosage effect of Pax9 in the absence of Pax1, with increasing severity of the VC malformations, whereby Pax1−/−Pax9−/− mutants exhibit a complete loss of VBs and IVDS, deformed proximal parts of the ribs and a lack of caudal vertebrae. Sclerotomal cell proliferation is significantly reduced in the Pax1−/− and Pax1−/−Pax9−/− embryos, while Pax1 and Pax9 are believed to be essential for proliferation, but not needed for sclerotome formation. Moreover, they are postulated to have a role in early chondrogenesis in axial skeleton via regulation of processes vital for condensation of the prechondrogenic mesenchyme, such as control of cell shape/size, cell adhesion, and extracellular matrix (ECM) reorganization (Peters et al., 1999; Wallin et al., 1994).

While the functions of Pax1/Pax9 have been hypothesized based on the phenotypic outcomes of their knock-outs, their true molecular functions, target genes and mutual regulation (or compensation) in IVD development are largely unknown. Notably, their role in axial skeletogenesis is conserved in mice and humans. Besides the high similarity of the paired-domain sequence between murine and human Pax genes, mutations or deficiency of PAX1 and/or PAX9 have been associated with Jarcho–Levine and Klippel–Feil syndromes which are characterized by kyphoscoliosis or vertebral segmentation defects that phenocopy the Pax1−/−Pax9−/− mouse mutants (Bannykh et al., 2003; Lopez et al., 1997). Therefore, deciphering the molecular roles of Pax1 and Pax9 would be valuable to understand the basis of human vertebral defects.
Here, we have identified for the first time, the in vivo targets of Pax1 and Pax9 in IVD anlagen cells to elucidate their roles during the early stages of IVD development by using enhanced green fluorescent protein (EGFP) gene targeting, fine tissue dissection and fluorescence-activated cell sorting (FACS) for the specific isolation of Pax1- and Pax9-expressing cells. Using multiple allele KO embryos we identified Pax1 and Pax9 targets that were obscured in the single mutant embryos by the functional redundancy of Pax1/Pax9. Consistent with prior hypotheses, Pax1/Pax9 have a role in regulating the early functions of IVD morphogenesis such as cell proliferation, adhesion, cell motion, mesenchyme condensation and regulating the early functions of IVD morphogenesis such as cell loss of Trends in gene expression changes of target genes with increasing fibrillogenesis and cartilage development, were also revealed. Fluorescence activated cell sorting (FACS) for the specific isolation embryos we identified Pax1 and Pax9 targets that were downstream targets of Pax1/Pax9, namely collagen fibrillogenesis and cartilage development, were also revealed. Trends in gene expression changes of target genes with increasing loss of Pax1/Pax9 alleles revealed potential mechanisms by which they compensate for each other. Especially, loss of Pax9 is likely compensated for by the increased Pax1 expression. Remarkably, 41 of the differentially expressed genes are associated with relevant axial skeletal defects. Moreover, several genes known to be regulated by the Sox trio (Sox5/Sox6/Sox9), important regulators of chondrogenesis in IVD development (Han and Lefebvre, 2008; Smits and Lefebvre, 2003), were found to be downstream targets of Pax1 and Pax9 as well. In vivo high-throughput chromatin immunoprecipitation-sequencing (ChIP-Seq) revealed critical cartilage development genes to be directly regulated by Pax9 including Sox5 itself. In addition, Pax1/Pax9 are connected with BMP and TGF-B pathways in IVD development. Finally, we also show that Pax1/Pax9 and Sox5 are connected by a negative feedback loop providing a potential mechanism for Pax gene down-regulation in the mature IAF of the IVD, and so segregating the IAF and OAF. In conclusion, we propose that Pax1 and Pax9 initiate robust expression of early chondrogenic genes during the earliest phase of mesenchymal differentiation in IVD development, highlighting how it is finely regulated spatio-temporally by complex transcriptional feedback loops.

RESULTS

Pax1-EGFP and Pax9-EGFP mouse lines for isolating Pax-specific population of cells from embryonic IVD

To facilitate the isolation of Pax1- and Pax9-expressing cells from mouse embryonic tissues, we generated three knock-in transgenic mouse lines under the control of a Pax1 or Pax9 regulatory element. We used a 2A-peptide strategy to co-express EGFP downstream of a functional Pax1 protein (Fig. S1A-C). Similarly, the paired-box domain in the exon 2 of Pax1 and Pax9 was disrupted to generate the Pax1 and Pax9 knockout (KO) mouse lines (Fig. S1D-F). The generation and characterization of the Pax1EGFP;Pax9−/− KO line (Pax1−/−) has been described already (Sivakamasundari et al., 2013). Abbreviations for mouse lines used in the text henceforth are summarized in Fig. 1A. The Pax1F2A−:EGFP::Pax1− (hetero- and homozygous, henceforth referred to as Pax1WT::EGFP and Pax1WT::EGFP++, respectively) and Pax9+/−:EGFP::Pax9−/− (called henceforth Pax9+/−/−) mice were viable and fertile while the Pax9EGFP::Pax9−/−::EGFP::Pax9−/− (called henceforth Pax9−/−/−) exhibited post-natal lethality as previously reported. These mouse lines expressed EGFP in the correct Pax1- or Pax9-specific domains (Fig. S2). Immunohistochemistry (IHC) analyses confirmed the absence of Pax9 protein in the Pax9−/−/− embryos (Fig. S3C).

In order to investigate the functions of Pax genes and their dosage compensation during IVD development, EGFP-positive cells were isolated from dissected vertebral column (VC) tissue at E12.5 via FACS and subsequent gene expression analyses were performed (Fig. 1B). In the population of VC cells isolated from E12.5 Pax1WT−:EGFP, Pax9+/− and Pax9−/− embryos, Pax1 wild-type or mutant transcripts were highly enriched in the EGFP(+) vs EGFP(−) cells, indicating a successful enrichment for the correct cell population (Pax1 expressing) from these embryos (Fig. 1C and Table S1). Pax9 was also enriched in these EGFP(+) cells albeit not to the same extent as Pax1 (Fig. 1C). Gene expression profiling of EGFP(−) versus EGFP(+) cells from Pax1WT−:EGFP revealed 744 down-regulated and 1052 up-regulated genes in the EGFP(+) cells relative to the EGFP(−) cells (Table S1; fold change (FC) ≥1.5 and P<0.05). Gene ontology (GO) enrichment analysis revealed ‘biological processes’ categories such as extracellular matrix (ECM), cell adhesion, cartilage development, collagen fibril organization, pattern specification, chemotaxis, cell shape and cell division to be over-represented. Genes involved in Wnt, TGF-B and hedgehog signaling pathways were also enriched (Fig. 1D) (DAVID; Huang et al., 2009b). Several other genes known to be expressed in the IVD anlagen or involved in the sclerotome differentiation and/or IVD development, such as Meox1, Sox5, Foxe2, Adams12, Fmod and Tgfbr2, were enriched in EGFP(+) cells (Table S1) (Furumoto et al., 1999; Mankoo et al., 2003; Smits and Lefebvre, 2003; Sohn et al., 2010).

Pax1 and Pax9 regulate proliferation, mesenchyme condensation and IVD development genes

At E12.5 the only morphological defects apparent in the Pax1+/− embryos were a loss of VB and IVD cells mainly in the lumbo-sacral region owing to partially redundant functions of the paralogous gene Pax9 (Sivakamasundari et al., 2013; Wilm et al., 1998). Analysis of Pax1+ cells from the E12.5 Pax1+/− vs Pax1WT−:EGFP IVD anlagen, revealed 50 down- and 80 up-regulated genes in Pax1−/− (FC≥1.5 and P<0.05) (Fig. 1E; Table S2). Down-regulated genes were enriched for ECM, while genes involved in organelle organization, cell adhesion, mesenchymal cell differentiation, transcriptional regulation and cell migration were up-regulated (Fig. 1F).

We subsequently analyzed the Pax1−/−Pax9+/− and Pax1−/−/−Pax9+/−/− compound mutants to uncover the obscured targets of Pax1 and Pax9. There were 599 genes differentially expressed (326 down; 273 up) in Pax1−/−Pax9+/− (versus Pax1WT−:EGFP) embryos. This is a 4.6-fold increase in the number of affected genes compared to Pax1−/−. In Pax1−/−Pax9+/− versus Pax1WT−:EGFP, 467 genes were differentially expressed, consistent with the less severe VC phenotype of Pax1−/−Pax9+/− mutants (Fig. 1E; Fig. S2, Table S3) (Peters et al., 1999). GO terms enriched in the down-regulated targets of Pax1−/−Pax9+/− include oxidative phosphorylation, cartilage development, apoptosis and stem cell maintenance. Notable GO terms over-represented in up-regulated targets were cell adhesion, blood vessel development, cell motion, skeletal muscle tissue development, GPCR signaling and cell-cell signaling. Common categories significantly enriched in both up- and down-regulated targets were ECM organization and collagen fibril organization (Fig. 2A,B). Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) also predicted ‘proliferation of cells’ to be decreased (z score=−2.524) and 85 proliferation-associated genes were differentially expressed in Pax1−/−Pax9+/− versus Pax1WT−:EGFP, consistent with a significant shortening of the tail in the E12.5 Pax1−/−Pax9+/− embryos, which worsened during later developmental stages (Fig. 2C; Table S4, Fig. S2-O). This corroborated prior findings of a significant decrease in proliferation in the tail region of E12.5 Pax1−/−Pax9+/−
embryos (Peters et al., 1999). In addition, for the first time, we describe a mis-localization of the normally ventro-medially located Pax1/Pax9-specific cells to the lateral regions of the VC at E14.5. No complete VBs or IVD structures were seen at this stage (Fig. 2C).

‘Developmental Disorder, Cell Morphology, Cellular Assembly and Organization’ was the top associated network (score=40) in IPA, with 83 genes associated with ‘Developmental Disorder’ ($P=1.76E^{-04}$–$2.77E^{-02}$) and 19 genes associated with ‘Connective Tissue Disorders’ ($P=3.91E^{-04}$–$2.77E^{-02}$) (Fig. 2D; Table S4); 41 of the differentially expressed genes were associated with relevant skeletal defects seen in the Pax1$^{-/−}$Pax9$^{-/−}$ mutants, such as kyphosis, scoliosis, lack of IVD, abnormal morphology of VC/tail/cervical vertebrae/sternum and chondrodysplasia (Fig. 2E; Tables S4 and S5). These results suggest that Pax1 and Pax9 regulate genes involved in processes essential for proper mesenchymal condensation – cell adhesion, ECM organization and cell migration during IVD development (Hall and Miyake, 2000).

Fig. 1. Experimental workflow and differentially expressed genes in Pax1/Pax9 mutants. (A) Abbreviations of Pax1 and Pax9 EGFP-expressing mouse lines. (B) Diagrammatic representation of experimental workflow. (C) Pax1 and Pax9 fold enrichment in EGFP$^{+}$ cells compared to EGFP$^{−}$ cells from E12.5 embryos. (D) Bar chart of enriched GO terms in E12.5 Pax1 EGFP$^{+}$ cells. (E) Number of differentially expressed genes for the various genotype comparisons. (F) Bar chart of enriched GO terms for down-regulated and up-regulated genes in E12.5 Pax1$^{-/−}$ embryos. GOI, gene of interest; EGFP, enhanced green fluorescent protein; FACS, fluorescence activated cell sorting; GO, gene ontology; WT, wild type.

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Gene dosage effect of Pax1 and Pax9 at molecular level

To understand the gene dosage effect of Pax1 and Pax9 at a molecular level, we analyzed the trends in expression changes of their targets upon gradual loss of Pax1 and Pax9 alleles. Majority of the genes could be categorized into three main groups (Fig. 3A-C).

Group 1 (118 down, 108 up) genes showed a gradual decrease or increase in expression with increasing loss of Pax1/Pax9 alleles, whereby a significant change in expression level (FC$>1.5$) occurred only upon the loss of all four alleles. This was consistent with the observed increasing severity of the mouse phenotype with increasing loss of Pax1 and Pax9 alleles. Genes found in this group were mostly ECM-related and included Acan. Notably, chromatin remodeling and acetylation factors like Ep300 and Phf20 were also found in this category (Fig. 3A; Tables S5 and S6).

Group 2 (166 down, 109 up) consisted of genes which showed a significant change only upon the loss of three alleles ($Pax1^{−/−}Pax9^{+/−}$), but no further significant change upon the loss of only one more allele. Genes found in this group were mostly associated with the respective axial skeletal defects, identified via IPA (top) and MGI phenotype (bottom). Genes down-regulated in $Pax1^{−/−}Pax9^{−/−}$ respectively to WT are in blue. GO, gene ontology; VC, vertebral column; IVD, intervertebral disc anlagen.

![Gene ontology and Ingenuity Pathway Analyses (IPA) of differentially expressed genes in Pax1/Pax9 mutants.](image)

(A,B) Bar chart of enriched GO terms for down-regulated (A) and up-regulated genes (B) in E12.5 Pax1$^{−/−}Pax9^{−/−}$ embryos. (C) Pax1$^{−/−}Pax9^{−/−}$ embryos exhibited shortened tail phenotype compared to Pax1$^{−/−}$ embryos. Yellow arrows indicate tail tip. Fluorescence was seen in the IVD of E14.5 Pax1$^{−/−}Pax9^{+/−}$embryos in a regular metameric fashion. In the Pax1$^{−/−}Pax9^{−/−}$ embryos, the fluorescing cells were mis-localized to the sides of the embryo with the notochord exposed in the middle (orange arrow). White arrows indicate Pax1/Pax9 lineage cells expressing EGFP in the IVD, detected by the presence of GFP expression. (D) Number of differentially expressed genes in E12.5 Pax1$^{−/−}Pax9^{+/−}$ that are associated with the respective axial skeletal defects, identified via IPA (top) and MGI phenotype (bottom). Genes down-regulated in Pax1$^{−/−}Pax9^{−/−}$ respective to WT are in blue. GO, gene ontology; VC, vertebral column; IVD, intervertebral disc anlagen.
of the last copy of Pax9 (Pax1−/−Pax9−/−). This suggests that two copies of Pax9 were essential to maintain the normal expression level of these genes in the absence of Pax1. This group encompassed oxidative phosphorylation genes among other ECM genes like Hspg2 and Cspg2 and cartilage development gene Bmp4 (Fig. 3B; Tables S5 and S6).
Group 3 (28 down, 31 up) genes showed a significant change only upon the loss of the last copy of Pax9 in the absence of Pax1. Intriguingly, this included several key cartilage development and collagen fibrillogenesis genes such as Col2a1, Sox5, Wwp2, Sim2, Ctgf and Dpt (Fig. 3C; Tables S5 and S6). These genes could be too crucial to be compromised, hence maintained at appropriate levels even by the last copy of Pax9.

**Regulation of Pax1 and Pax9**

We next examined the expression levels of Pax1 and Pax9 in the various mutants to understand their compensation mechanism. Pax1 was down-regulated in Pax1+/− mutant, but Pax9 remained unchanged in Pax9−/− (Fig. 3D; Table S4). This indicated that Pax1 could auto-regulate itself, unlike Pax9. Indeed, Pax1 was down-regulated in the comparison between Pax1+/−/Pax9+/− and Pax1+/−/Pax9−/−, revealing that a single copy of Pax1 could regulate itself (Fig. 3F); however, Pax9 was not considerably altered in any of the compound mutants (Fig. 3E,F; Table S3). This corroborates prior observations that Pax9 expression is not dependent on Pax1 or Pax9 (Neubüser et al., 1995).

Pax1 did not significantly change between Pax1+/−/Pax9+/− and Pax1+/−/Pax9−/− (Fig. 3E; Table S3). Moreover, there was only a slight decrease (1.5-fold) in Pax1 between Pax1+/−/Pax9+/− and Pax1+/−/Pax9−/− (Table S3). This suggested that at least two copies of Pax9 was required to even have a minute effect on Pax1. We also did not observe any significant change in Pax9 level or spatial expansion of its expression domain in Pax1−/− mutants based on microarray (Table S2) and IHC analyses (Fig. S3). Yet, Pax9 expression was significantly up-regulated in E13.5 Pax9−/− mutants (Fig. 3D).

Together these data suggest that Pax9 is unable to compensate for the loss of Pax1 by up-regulating itself at E12.5 and E13.5, while Pax1 may compensate for the loss of Pax9 by altering its own expression level via a positive auto-feedback mechanism.

**Pax1 and Pax9 redundantly regulate key cartilage development genes**

Closer inspection of genes regulated by Pax1 and Pax9 revealed key cartilage development genes like Sox5, Acan, Col2a1 and Wwp2 to be within the top 30 positively regulated targets of Pax1 and Pax9. KO mutants of these four genes are known to exhibit axial skeletal and craniofacial defects similar to Pax1+/−/Pax9−/− mutants (Aszódi et al., 1998; Smits and Lefebvre, 2003; Watanabe et al., 1997; Zou et al., 2011).

Removal of the last copy of Pax9 in the absence of Pax1 (Pax1+/−/Pax9+/− vs Pax1+/−/Pax9−/−) revealed its positive regulatory effect on Sox5, Acan, Col2a1 and Wwp2 (Fig. 3E; Table S3). Quantitative PCR (qPCR) of FACS-sorted cells of Pax1+/−/Pax9+/− and Pax1+/−/Pax9−/− showed a similar trend, whereby all four targets were positively regulated by a single copy of Pax1 in the absence of Pax9, affirming that both Pax1 and Pax9 were independently capable of up-regulating these targets (Fig. 3F). We validated some of these targets by sectioned in situ hybridization (Fig. S5.2).

While these targets are also known to be positively regulated by Sox9 (Akiyama et al., 2002; Bell et al., 1997; Han and Lefebvre, 2008; Nakamura et al., 2011), a key regulator of chondrogenesis, we did not observe significant changes in Sox9 expression levels in Pax1+/−/Pax9+/− versus Pax1+/−/EGFP (Table S3) and Pax1+/−/Pax9−/− versus Pax1+/−/Pax9+/− comparisons (Fig. 3F). This suggests that the observed in vivo changes in expression levels of these genes are Pax1- and Pax9-dependent and not caused by changes in Sox9 levels, indicating the existence of a potential parallel Sox9 independent mechanism.

**Pax9 directly regulates chondrogenic genes essential for IVD morphogenesis**

These observations prompted us to inspect if Pax9 was capable of directly regulating these critical cartilage development and collagen fibrillogenesis genes to compensate for the loss of the Pax1.

Therefore, we assessed the in vivo binding profile of Pax9 by chromatin immunoprecipitation sequencing (ChIP-Seq) on dissected E12.5 WT VC tissues. There were 11,133 Pax9 binding loci which were associated with 6380 genes (Table S7). Majority of the Pax9 binding sites were found in the distal (29.4%), followed by intragenic (24.9%), TSS (20.8%), >100 kb from TSS region (17.0%), proximal (4.5%) and promoter regions (3.4%) (Fig. 4A).

GO analysis revealed that the genes associated with Pax9 binding sites were enriched for Pax9 expression domains: maxilla, vertebral cartilage, chondrocranium, paraxial and forelimb mesenchyme; and the relevant mouse mutant phenotypes: abnormal dentin/pterygoid process, abnormal palate shelf elevation, abnormal sternebra morphology and paraxial mesoderm and polysyndactyly (Fig. 4B) (McLean et al., 2010). De novo motif discovery identified a Pax9 motif, 5’ CGCGTGACC 3’, that resembled the previously reported 3’ half site for the Pax family of TFs (Fig. 4C) (Czerny et al., 1993). Analysis using Centrimo also revealed Pax DNA binding domains (BDB) to be centrally enriched (Fig. S4A,B) (Bailey and Machanick, 2012). It is noteworthy that the Pax DBDs are highly similar, so various Pax TFs appear to be enriched.

In addition, 24.4% (146 genes) of the differentially expressed genes in Pax9−/− had Pax9 binding sites associated with them. This corresponds to 24.8% of the down-regulated and 23.8% of the up-regulated targets (Fig. 4D). Of the down-regulated genes, nine of them were directly regulated by a single copy of Pax9 while 38 were regulated by two copies of Pax9 (Fig. 4D). This included Col2a1, Wwp2, Clx2, Hip1, Sox5 but not Acan, indicating that the latter is an indirect target of Pax9. These binding regions also possessed the Pax9 de novo motif (P<0.005) (Fig. 4E). Since Pax9 is capable of directly regulating these genes it explains why their expression levels do not significantly change in the absence of only Pax1, and reveals how it compensates for these genes (Fig. 3A-C,E).

**Sox5 and Sox6 negatively regulate Pax1 in IVD anlagen cells**

Sox5 and Sox6 are important in IVD morphogenesis, whereby they play redundant but important roles in up-regulating major cartilage ECM genes like Acan and Col2a1 for the timely maturation of chondroblasts and promoting inner anulus differentiation (Smits and Lefebvre, 2003). Also, Sox5 is co-expressed with Pax1/Pax9 in the VC at E12.5 and E13.5 (Fig. 5; Table S1). Hence, we investigated previously generated Sox5+/- and Sox6+/-/EGFP WT and KO mutants (data not shown) made in an identical manner to the Pax1/Pax9 mutants. (GSE33173; Fig. 4F; Table S8). This suggests a synergistic repressive effect of both Sox genes on Pax1, while Pax9 expression was unchanged in the Sox5+/− or Sox6−/− or Sox5−/− Sox6−/− mutants.

Considering that Pax1 and Pax9 have an activating effect on Sox5, and Sox5/Sox6 synergistically repress Pax1, and positive regulation of Sox5/Sox6 by Sox9 is well-established (Akiyama et al., 2002), we hypothesize that these Pax and Sox genes are connected in a negative feedback loop during VC development (Fig. S5). We posit that Pax1/Pax9 are downregulated in the cartilaginous IAF by this negative feedback mechanism, possibly playing a role in the segregation of IAF from OAF by E15.5.
Pax1/Pax9 regulation of IVD development is intertwined with BMP and TGF-B pathways

We found both Pax1/Pax9 and Sox5/Sox6 regulated Bmp4 indicating the involvement of BMP pathway in IVD development (Fig. S5.2A).

A study by Sohn et al. (2010) showed that BMP signaling activated the Sox trio and cartilage genes (Acan, Wwp2) in the sclerotome, while TGF-B signaling regulated IVD markers (Fmod and Adamts12) and maintained the boundary between vertebrae and IVD in the axial.
skeleton (Sohn et al., 2010). Also, studies on Tgbr2 conditional KO mutants have shown that TGF-B signaling is required to define the boundary between VB and IVD and to promote differentiation of annulus fibrosus from the sclerotome (Baffi et al., 2006, 2004; Sohn et al., 2010). Therefore we probed in our data if Pax1/Pax9 regulate Bmp4 or TGF-B regulated genes.

Using data from Sohn et al. (2010) performed at E12.5, we compared our list of Pax1/Pax9 regulated genes with the list of Tgfb3 and Bmp4 in the IVD anlagen at E12.5. (J-M) Expression of Pax1, Pax9, Sox5 and Sox9 in the IVD anlagen at E13.5. (N-R) Expression of Pax1, Pax9, Sox9 at E15.5 and Tgfb3 and Bmp4 at E14.5 in the IVD. (S) Proposed negative feedback loop mechanism between Pax, Sox, Bmp4 and TGF-B pathway at E12.5. Black arrows indicate the final site of expression at E14.5. Brackets indicate IVD region. VB, vertebral body; n, notochord; IVD, intervertebral disc; NP, nucleus pulposus; IAF, inner annulus fibrosus; OAF, outer annulus fibrosus; WT, wild type; SISH, sectioned in situ hybridization; D, dorsal; V, ventral.

Fig. 5. IVD expression of Pax and Sox genes and proposed model. (A) Sagittal section of E13.5 WT vertebral column showing Pax1 expression in the IVD anlagen detected by SISH assay. (B) Mallory’s tetrachrome staining of E15.5 WT vertebral column; IAF is stained blue and OAF is stained dark red. (C,D) Immunohistochemistry and Alcian blue staining of vertebral column showing (C) Pax1 and (D) Pax9 protein expression restricted to the OAF at E15.5. (E-I) Expression of Pax1, Pax9, Smad3, Tgfb3 and Bmp4 in the IVD anlagen at E12.5. (J-M) Expression of Pax1, Pax9, Sox5 and Sox9 in the IVD anlagen at E13.5. (N-R) Expression of Pax1, Pax9, Sox9 at E15.5 and Tgfb3 and Bmp4 at E14.5 in the IVD. (S) Proposed negative feedback loop mechanism between Pax, Sox, Bmp4 and TGF-B pathway at E12.5. Black arrows indicate the final site of expression at E14.5. Brackets indicate IVD region. VB, vertebral body; n, notochord; IVD, intervertebral disc; NP, nucleus pulposus; IAF, inner annulus fibrosus; OAF, outer annulus fibrosus; WT, wild type; SISH, sectioned in situ hybridization; D, dorsal; V, ventral.
genes regulated by BMP and TGF-B pathway. We found some of the BMP and TGF-B pathway genes were also regulated by Pax1/ Pax9 (Fig. 4G; Table S9). Importantly, Sox5, Acan and Wwp2 were activated by both Pax1/Pax9 and BMP pathway, while Papss2 was activated by BMP, TGF-B pathways and Pax1/Pax9 (Fig. 4G; Table S9). Also, VB enriched genes identified by Sohn et al. (2010) were repressed by both Pax1/Pax9 and TGF-B (Ebfl, Gdf10, Alcam, Nr2f2). Indeed, from our Pax9 ChIP-seq data, we found Smad1 and Smad2 motifs to be centrally enriched (within 100 bp) in the Pax9 bound regions (Fig. 5A-C) (Bailey et al., 2009; Bailey and Machanick, 2012). Also, Smad3, Tgfl3 and Bmp4 were all expressed in the IVD anlagen at E12.5 (Fig. 5). This affirmed that Pax1/Pax9, BMP and TGF-B pathways were interlinked in the regulation of some of the IVD genes.

The distinction between IAF and OAF happens only at E14.5 onwards, and is more apparent at E15.5 (Fig. 5A-D). To assess whether Pax, BMP and TGF-B pathways played a role in the distinction between IAF and OAF, we performed some sectioned in situ hybridization (SISH) and also mined the database of gene expression at E14.5 (EUExpress database, www.eurexpress.org; Dzie-Roux et al., 2011). Among the 41 genes regulated by Pax1/ Pax9, TGF-B and Bmp4, a majority of those activated by Pax1/Pax9 at E12.5 were expressed in the IAF later at E14.5, while those repressed were mainly expressed in OAF and VB at E14.5 (Table S9, EUExpress database). On the contrary, several of the targets activated at E12.5 by Pax1/Pax9 alone were expressed highly in the E14.5 OAF as well (Fig. S5.1, Fig. S5.2). These indicated that Pax1/Pax9 on their own were capable of activating both IAF and OAF genes, while in combination with BMP and TGF-B pathways, and were mainly involved in promoting IAF.

Notably, Pax1/Pax9, Bmp4 and Tgfl3 were restricted to OAF, while Bmp2, Tgflr1, Smad2 and Smad3 were all expressed in both IAF and OAF at E14.5-E15.5, suggesting that Pax1/Pax9, BMP and TGF-B signaling were active in OAF at E14.5 and may play a role in further OAF differentiation (Fig. 5; EUExpress database; Table S9).

DISCUSSION

The importance of Pax1 and Pax9 in axial skeletogenesis was demonstrated more than a decade ago using KO mouse models, but not as much progress has been made since to decipher their molecular roles (Hall and Miyake, 2000). Our genome-wide study is the first to identify the in vivo target genes of Pax1 and Pax9 in the early embryonic IVD in a cell-type-specific manner. The E12.5 developmental stage was chosen as this is when the IVD anlagen is first formed. We have identified genes coexpressed in Pax1 expressing cells to be involved in the Wnt, Tgf-B and hedgehog signaling pathways. These pathways are critical in a vast number of cellular processes relevant to the formation of mesenchymal condensations and chondrogenesis/osteogenesis in early IVD development (Buttitta et al., 2003; Ling et al., 2009; Sohn et al., 2010). Furthermore, our analysis of Pax1−/− and Pax1/Pax9 compound mutants revealed the roles of Pax genes in processes essential for mesenchymal condensation – a prerequisite for the formation of skeletal elements via endochondral ossification well-known to be disrupted in Pax mutants (Wallin et al., 1994).

The small number of differentially expressed genes in the E12.5 Pax1−/− embryos indicated that the complete repertoire of targets were being masked because of compensation by Pax9, consistent with the mild phenotype whereby a shortening of tail was obvious only from E13.5 onwards (Sivakamasundari et al., 2013). Analysis of the compound mutants embryos revealed several more targets of Pax1 and Pax9, fitting the observed severity of the phenotype with increasing loss of Pax1 and Pax9 alleles. One of the striking defects reported for Pax1−/−Pax9−/− mutants were reduced cell proliferation in the lumbo-sacral region (Peters et al., 1999). In our study, a significant number of cell proliferation genes were affected only upon the KO of 3/4 or 4/4 (all) alleles but not in the Pax1−/− (2/4 alleles). This correlates with the shortened tail, evident as early as E12.5, and the loss of the medially located IVD and VB cells in the double-null mutants. These defects already prefigure the future adult Pax1−/−Pax9−/− mutants, which exhibit a complete lack of sacral elements, IVD or VB structures (Peters et al., 1999). Alterations to the cell-adhesion and cell motion genes may also play a role in the observed mis-localization of Pax1/Pax9 cells to the lateral regions of the VC in the Pax1−/−Pax9−/− embryos. Besides proliferation, oxidative phosphorylation genes were affected in the double-null mutants. It has been shown that disruption of oxidative phosphorylation affects cell viability and proliferation in neural progenitors (Lee et al., 2013). It is likely that this could have partly contributed to the overall decrease in proliferation and/or cell viability of Pax1/Pax9 deficient cells. Highly proliferating cells such as the cells of the somites are believed to be particularly sensitive to changes in rates of protein synthesis (Oliver et al., 2004).

Down-regulation of several ribosomal genes may have an effect on translation in general, which could also have contributed to the reduced cell proliferation. Furthermore, we found several of the Pax1/Pax9 regulated genes to be associated with axial skeleton defects in humans that phenocopy the Pax1−/−Pax9−/− mouse mutants. For instance, the human ortholog of Acan, Col2a1, and Col11a1 are linked to spondyloepiphyseal dysplasia, osteoarthritis, disc degeneration (Nakane et al., 2011; Sahlman et al., 2001), and susceptibility to lumbar disc herniation (Mio et al., 2007).

Beyond understanding the molecular roles of Pax1 and Pax9, we aimed to address how they compensate for each other. We have uncovered the gene dosage effect of Pax1 and Pax9 at a molecular level by identifying the groups of genes requiring all four copies of Pax1/Pax9, two copies of Pax9 or only one copy of Pax1 or Pax9. We delved deeper to understand if expression of Pax1 and Pax9 themselves changed to cater to the dosage compensation. To maintain the required dosage of Pax proteins for gene regulation, it is expected that Pax1 or Pax9 should be up-regulated in the absence of the other protein. We observed that Pax1 is up-regulated in the absence of Pax9, suggesting that it may compensate for the required dosage of Pax by up-regulating its expression via auto-regulation. Only upon the loss of one more copy of Pax1 (Pax1+/−Pax9−/−), its dosage likely becomes critical. With only one copy of Pax1, the system may be sufficiently deprived by diminishing levels of Pax1 being produced, thus resulting in the onset of more severe vertebral defects in Pax1+/−Pax9−/−; however, Pax9 was not up-regulated in the absence of Pax1 protein which could explain its inability to completely rescue VC defects in Pax1−/−. These observations support the existing hypothesis that Pax1 is the more dominant player since it fully recruits the vertebral phenotype in the Pax9−/− mutant, unlike Pax9 (Peters et al., 1999). We postulate that in the absence of Pax1, Pax9 maintains the levels of vital IVD morphogenesis genes (Sox5, Col2a1, Wwp2) owing to its ability to directly regulate them. Also, we speculate that Pax1 and Pax9 may be capable of binding to each other’s sequences, as their paired domains are highly similar with only three amino acid changes (Neubüser et al., 1995). In such a scenario, competition would exist between Pax1 and Pax9 under wild-type conditions, whereby the amount of protein, partner proteins (cofactors), chromatin...
accessibility and/or the time at which the proteins are expressed may determine whether Pax1 or Pax9 bind the specific site and their affinity in binding. Temporal differences in the emergence of Pax1 and Pax9 expression exist, whereby Pax1 is expressed in the de-epithelializing somites at a slightly earlier time-point (E8.5) than Pax9 (E9.0) (Neubüser et al., 1995; Wallin et al., 1994). This initial period could be critical in development which is often a highly precisely timed event in mammals. Targets that are initiated early by Pax1 could be critical for subsequent gene regulation, such as through the priming of the chromatin landscape or generating essential co-factor proteins required for later regulatory steps. For example, chromatin remodeling and acetylation factors like Ep300, Phf20 and Hdac2 all gradually declined or increased with the loss of Pax1 and Pax9. As a result, although Pax9 expression is not dependent on Pax1, it may not be able to fully compensate owing to the incomplete chromatin priming and/or lack of initiation of the required co-factor proteins. Certainly these postulations warrant further studies which would illuminate a more detailed mechanism of compensation by these TFs. Performing ChIP-Seq on reciprocal null backgrounds, and identification of Pax1 binding sites in the wild type would help to clarify this hypothesis. Lack of a good ChIP-grade antibody against Pax1 precluded our analysis of Pax1 binding sites in this study.

Remarkably, analysis of our Pax1/Pax9 regulated targets and Sox5/Sox6 targets in the IVD revealed the existence of a negative feedback loop between the Pax and Sox genes. Because Pax1 and Pax9 are known to be down-regulated once the pre-chondrogenic cells mature into chondrocytes in the inner annulus (IAF), and the Sox trio are essential for and are up-regulated during chondrogenesis (Balling et al., 1988), this negative feedback circuit might explain the initial co-expression of the Sox and Pax genes in the IVD mesenchyme at E12.5–E13.5 and the subsequent restriction of Pax1/Pax9 to the OAf by E14.5–E15.5. While Pax-Sox relationship is well known in neurogenesis whereby Sox6 and Sox1/Sox2 are known to cooperate, here we have identified the existence of such a regulatory axis involving Pax9 and Sox in IVD development (Curto et al., 2015). Importantly, this Pax-Sox regulation might have a key role in the segregation of IAF and OAF.

Moreover, Pax1 and Pax9 positively regulate Bmp4 and several of the cartilage genes activated by the Sox trio. Indeed, Bmp4 is an upstream regulator of the Sox trio (Sohn et al., 2010). This prompted us to explore the involvement of BMP pathway in IVD development. Mining the publication by Sohn et al. (2010) revealed that Pax1/Pax9 regulate genes controlled by BMP and TGF-B pathways in the IVD. At E12.5, Pax1/Pax9 positively regulate both IAF and OAF genes, while in combination with BMP and TGF-B pathways they appear to be activating IAF genes. We thus posit that Pax1/Pax9, BMP and TGF-B pathways together are mainly involved in promoting cartilaginous inner annulus development and preventing inappropriate VB or fibrous annulus development. However, the restriction of Pax1/Pax9, Bmp4 and TGF-B components to the OAF at E14.5 onwards indicate that they may be involved in subsequent embryonic OAf differentiation. Besides, other signaling pathways may also play a role in this complex process, as the FGF pathway effector, Etv1, was downstream of Pax1/Pax9 and is restricted to OAf at E14.5.

Based on our findings, we propose that Pax genes assist in the activation of early chondrogenic genes including Sox5 and Bmp4 during the IVD mesenchyme differentiation. In turn, Sox5 represses Pax1 and Bmp4 by negative feedback, allowing the Sox trio to take full control of the chondrogenic program. In support of this, the Sox5−/−Sox6−/− IVD mesenchymal cells, which still express Pax1, fail to undergo chondrogenesis and remain mesenchymal at E15.5 (Smits and Lefebvre, 2003). Moreover, a recent publication showed that misexpression of Pax1 in cultured chondrocytes resulted in downregulation of Sox9, Col2a1 and Acan, indicating its role as a negative regulator of chondrocyte maturation (Takimoto et al., 2013). While in our study Pax1/Pax9 positively regulate Col2a1 and Acan in prechondrogenic mesenchymal cells of the IVD, how it regulates these targets during later stages of in vivo chondrogenesis, particularly in the context of IVD anlagen, is unknown. Nevertheless, their observation of antagonistic relationship between Pax1 and Sox9 support our hypothesis that tipping the fine balance between Pax and Sox is essential to drive the prechondrogenic IVD anlagen cells towards a cartilaginous inner annulus followed by restriction of Pax and Bmp4 to the fibrous outer annulus in a timely manner.

Our novel findings in this study, of a complex Pax-Sox gene network that is interconnected with BMP and TGF-B pathways, illuminates an essential mechanism of the early IVD morphogenesis. Importantly, identification of Pax1 and Pax9 as regulators of critical IVD genes has implications in understanding certain forms of kyphoscoliosis that have been linked to human Pax1 and Pax9, as well as degenerative disc disease.

MATERIALS AND METHODS

Ethics statement

All animal procedures were performed according to the Singapore A*STAR Biopolis Biological Resource Center (BRC) Institutional Animal Care and Use Committee (IACUC) guidelines which are set by the National Advisory Committee for Laboratory Animal Research (NACLAR) Singapore. The IACUC protocols employed were reviewed and approved by the aforementioned committee before any animal procedures were undertaken for the study described here (IACUC Protocol No: 110689 and 110648). The mouse strains used in this study were housed, maintained and provided by the A*STAR Biopolis Biological Resource Center. The lines described here will be made available to the research community upon acceptance of the manuscript.

Gene targeting

Bacterial artificial chromosomes (BAC) clone RP24-88N2 and RP24-211H10 (derived from the C57BL/6J mouse strain) containing the Pax1 (chromosome 2) and Pax9 (chromosome 12) gene loci respectively were obtained from the BACPAC Resources Centre (CHORI) (bcapcresources.org). According to the manufacturer’s protocol, genetic modifications of the clones were performed using the Gene Bridges Quick and Easy BAC Modification kit (#K001, Gene Bridges, Heidelberg, Germany). Details of targeting constructs and mouse generation, and genotyping, Southern blotting, FACS sorting, microarray, ChIP-Seq, histology and primers are described below.

Targeting strategies using BAC recombinering technology, ES cell homologous recombination and mouse crosses

For the Pax1IF2A-EGFP;Pax1−/− construct, the RAKR-GSG-FT2A-EGFP-FRT-PGK-gb2-Neo-FRT cassette was inserted in frame immediately before the STOP codon at exon 5 of Pax1. For the Pax9IF2GFP;Pax9−/− construct, the same cassette was inserted at three amino acids after the start of exon 2 of Pax9. Successfully modified mutation-free BAC clones were subcloned as per the manufacturer’s protocol into a minimal vector using the Gene Bridges Quick and Easy BAC Subcloning kit (# K003, Gene Bridges). A Pmel restriction site was added at the end of the lower grabbing arm to facilitate subclone linearization. Linearized subclone was electroporated into R1 or V6.4 mouse embryonic stem cells (ESC) to generate the Pax1IF2A-EGFP;Pax1−/− and Pax9IF2GFP;Pax9−/− ES clones respectively (Eggan et al., 2001). Details of the generation of the Pax1IF2GFP;Pax1−/− construct has been described in (Sivakamasundari et al., 2013). The correctly targeted clones were confirmed via Southern blotting, karyotyped and microinjected into 8-cell
stage mouse embryos isolated from C57BL/6J mice (Kraus et al., 2010). Owing to postnatal or pre-natal lethality, heterozygote Pax9<sup>+/−</sup> mice were mated to generate Pax9<sup>−/−</sup> embryos while double heterozygote mice (Pax1<sup>+/−</sup>Pax9<sup>−/−</sup>) were mated to generate the Pax1<sup>−/−</sup>Pax9<sup>+/−</sup>, Pax1<sup>+/−</sup>Pax9<sup>−/−</sup> and Pax1<sup>−/−</sup>Pax9<sup>−/−</sup> embryos.

**Southern blotting and PCR genotyping**

Methods of genomic DNA extraction from the ESC clones and mouse tail and Southern blotting were described previously (Sivakamasundari et al., 2012). Genotyping details for Pax1<sup>EGFP:Pax1</sup> was performed as described in Sivakamasundari et al. (2013).

**Fluorescence Activated Cell Sorting (FACS) and RNA extraction**

Dissociation of mouse embryos into single cells was performed using enzymatic digestion with 0.05% Trypsin (Gibco), DNase (50 U/ml; Sigma) and Collagenase I & II (100 U/ml; Gibco), followed by filtration through a 100 μm mesh and then a 40 μm cell strainer before centrifugation at 448 g (2000 rpm) for 5 mins at 4°C. The cells were resuspended in 5% fetal bovine serum and 4 mM EDTA in Leibovitz’s L-15 medium for cell sorting using FACSARia flow cytometer (BD Biosciences) and collected into a 1.5 ml microcentrifuge tube containing 20% FBS buffer. Gating was performed using E12.5 WT embryos. Sorted cells were spun at 1400 g for 10 min at 4°C, resuspended in Trizol (Invitrogen) and incubated for 5 min at room temperature (RT). RNA was extracted using Trizol followed by column purification with the Qiagen RNeasy Micro kit, including on-column DNase treatment, RNA samples were quantified and checked for their integrity using Agilent RNA Pico 6000 Chip and Agilent 2100 Bioanalyzer software according to the manufacturer’s protocol. RNA was stored at −80°C until further use.

**Microarray**

Purified RNA samples with a RNA integrity number (RIN) value of at least 7.0 were chosen for subsequent cDNA conversion and linear single-round amplification using the NuGEN Ovation<sup>TM</sup> RNA Amplification V2 kit as per manufacturer’s protocol. Biotin labeling was performed with NuGEN Encore<sup>TM</sup> BiotinIL Module kit. An Illumina MouseWG-6 Expression BeadChip was used for microarray gene expression profiling, performed according to the manufacturer’s protocol, except that hybridization was performed at 48°C according to the recommendations by the NuGEN Ovation<sup>TM</sup> RNA Amplification V2 kit. A minimum of three biological replicates were used for each genotype for microarray analyses. Each biological replicate includes FACS-enriched cells from two finely dissected VCs so as to ensure sufficient RNA for downstream analyses.

**Gene expression analysis using GeneSpring GX 12.5**

Illumina® BeadStudio software was used to extract the raw image data from the scanned beadchips. The gene expression data was exported as sample probe profiles in a GeneSpring GX 12.5 compatible text file format, with background subtraction but no normalization. The text file was then imported into GeneSpring GX 12.5 (Agilent) for further gene expression analysis. The raw intensity data was quantile normalized to mitigate any batch effect. All entities were filtered by flags using default criteria in the scanned beadchips. The gene expression data was exported as sample IDs were used, classification stringency ‘medium’.

**Quantitative PCR**

Quantitative PCR was carried out using cDNA obtained by NuGEN OvationTM RNA Amplification V2 kit as mentioned above. Maxima SYBR Green/ROX qPCR Master Mix (2×) was used according to manufacturer’s protocol with Gapdh as endogenous control. qPCR was performed in triplicates and statistical analyses were performed in the Microsoft Excel using the data analysis tool (Analysis ToolPak).

**Gene dosage analysis**

All the differentially expressed genes (FC>1.5 or <−1.5) in the Pax1<sup>+/−</sup>Pax9<sup>−/−</sup> versus WT microarray were used in the gene dosage analysis. Microsoft Excel software was used to filter genes for the different groups according to the following criteria: Group1: genes not showing a FC>1.5 or <−1.5 for WT versus Pax1<sup>−/−</sup>, Pax1<sup>+/−</sup> versus Pax1<sup>−/−</sup>Pax9<sup>−/−</sup> or Pax1<sup>+/−</sup>Pax9<sup>−/−</sup> versus Pax1<sup>−/−</sup>Pax9<sup>+/−</sup>. Group2: genes not showing a FC>1.5 or <−1.5 for WT versus Pax1<sup>−/−</sup>, or Pax1<sup>+/−</sup>Pax9<sup>−/−</sup> versus Pax1<sup>−/−</sup>Pax9<sup>+/−</sup> but only in Pax1<sup>−/−</sup>Pax9<sup>−/−</sup>; and Group3: genes not showing a FC>1.5 or <−1.5 for WT versus Pax1<sup>−/−</sup>Pax9<sup>−/−</sup> or Pax1<sup>+/−</sup>Pax9<sup>−/−</sup> but only in Pax1<sup>−/−</sup>Pax9<sup>+/−</sup> versus Pax1<sup>−/−</sup>Pax9<sup>−/−</sup>. The normalized intensity values of the genes for each genotype (WT, Pax1<sup>+/−</sup>Pax9<sup>−/−</sup> and Pax1<sup>+/−</sup>Pax9<sup>+/−</sup>) were averaged across the respective biological replicates and plotted as intensity versus genotype for the respective groups.

**Chromatin Immunoprecipitation (ChIP-Seq)**

The VC tissues were dissected from staged (based on M. H. Kaufman morphological criteria (Kaufman, 1992) E12.5 mouse embryos in cold Leibovitz medium. The dissected tissues were homogenized and cross-linked with 1% cross-linking buffer (100 mM NaCl, 50 mM Hepes-KOH, ph 7.5, 1 mM EDTA, 0.5 mM EGTA, 11% formaldehyde) for 10 min at RT. Cross-linking was stopped with 0.25 M glycine and washed once with cold PBS and homogenized again. Nuclear extracts were obtained as per standard chromatin isolation protocol and chromatin was sheared to a size range of 100-500 bp. 2 mg of sheared chromatin was used for pre-clearing in a pre-washed anti-rabbit IgG antibody-conjugated Dynabeads® Protein G (ab46540; Invitrogen; #100.04D) in 0.5% BSA buffer for 1 h at 4°C. 1% of the pre-cleared sample was reserved as input. Immunoprecipitation (IP) was performed using pre-conjugated and pre-washed beads with anti-Pax9 antibody (Santa Cruz) for 24 h at 4°C. After the overnight IP, the beads were washed with wash buffer (50 mM Hepes, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate with 1× protease inhibitors) six times followed by one wash with TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 50 mM NaCl). The IP chromatin was eluted by incubation at 65°C for 30 min in 210 μl of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). The eluate and 1% of input that was reserved earlier were deskewed overnight at 65°C DNA, with sequential treatment of RNase A (0.2 mg/ml) and proteinase K (0.2 mg/ml) followed by DNA extraction by standard phenol: chloroform extraction method. DNA was quantified using Picogreen and 15 ng of purified IP or input DNA was used for library preparation using the NEBNext® ChIP-Seq Sample Prep Reagent kit. Size selection of 200-300 bp was performed on a 2% agarose gel and gel purified using QIAGEN gel purification kit. The ChIP DNA libraries were checked for their quantity and quality using Agilent Bioanalyzer DNA 1000 chip (85067-1504) before sequencing on Illumina’s Solexa Sequencer. Peak calling using MACS (Zhang et al., 2008), gene ontology and motif analysis methods using Weeder (Pavesi et al., 2004), MEME Suite (Bailey et al., 2009), Centrimo (Bailey and Machanick, 2012) and FIMO (Grant et al., 2011) are described below. Motif databases JASPAR CORE database (Mathelier et al., 2016), the UniPROBE database (Newburger and Bulyk, 2009), Human and Mouse HT-SELEX motifs from Jolma et al. (2013) and HOCOMOCO Human and mouse (v10) were used for motif analyses. Tracks for ChIP-seq peaks were visualized using Integrative Genomics Viewer (Robinson et al., 2011; Thorvaldsdottir et al., 2013) or UCSC genome browser (Kent, 2002).
Peak calling and motif analysis
After high-throughput sequencing of the short tags of DNA in the libraries, the sequence reads were mapped to the mouse genome (NCBI build 37/mm9). 11,133 peaks were called using Model-based Analysis of ChiP-Seq (MACS) algorithm (Zhang et al., 2008) with the default parameters and a P-value cut-off of 1.00E–10. The following criteria was used to assign the association of peaks to nearby genes: TSS, <1 kb upstream and downstream of the TSS from either side; promoter, 1-5 kb upstream of the TSS; intragenic, >1 kb downstream of TSS within the regions of the gene itself; proximal, 5-10 kb upstream of the TSS and 1-10 kb downstream of the TSS outside the gene; distal, 10-100 kb upstream and downstream of the TSS outside of the gene; others, >100 kb from TSS. The peaks called were ranked based on the P-value. De novo motif discovery was performed using Weeder (Pavesi et al., 2004) for the top 500 peaks with the lowest P-values. Only the central repeat-masked 200 bp sequences in each peak were used to achieve the best performance. Occurrences of the de novo discovered motif, Pax9 DBD, Pax1 DBD, Smad1 and Smad2 motifs were searched in all 11,133 peaks using FIMO (Grant et al., 2011) with a P-value threshold of 0.001. To identify the enrichment of binding sites of Pax9 and other cofactors, we used CentriMo (Bailey and Machanick, 2012) to scan each Pax9 ChiP-seq peak region (central 500 bp). De novo discovered Pax9 motif and all motifs in the JASPAR CORE database (Mathelier et al., 2016), the UniPROBE database (Newburger and Bulyk, 2009), Human and Mouse HT-SELEX motifs from Jolma et al. (2013) and HOCOMOCO Human (v10) and HOCOMOCO Mouse (v10), were used as input motifs. Gene Ontology (GO) enrichment analysis was performed using the Genomic Regions Enrichment of Annotations Tool (GREAT) web-based tool (http://bejerano.stanford.edu/great/public/html/). The default criteria was assigned to define the regulatory domain for this analysis: 5.0 kb upstream, 1.0 kb downstream, 1000 kb (1Mb) extension in both directions to the next closest gene’s TSS but a maximum extension only in one direction.

Genotyping PCR primers
PCR genotyping was performed using the following primers: 
Pax1P-5′CTTGTTGGAGATCTCAGT-3′; Pax1-5′AGATGAGTCTTGTAAGTCTTGACGGTAAGAGCCGACCACTTGGTA-3′; Pax9P-5′GGGTCTCTCTTCTTGTTTGTTGTT-3′; Pax9-5′ATCTAAAACCAAGACTCGGAAGAC-3′; 487 bp) Primer pair 1 (F – 5′ CTGTTGAGGATCTCAGTCC3′ and R – 5′ ATCTAAAAACCAAGACTCGGAAGAC3′; 444 bp) Primer pair 2 (F – 5′ CTGTTGAGGATCTCAGTCC3′ and R – 5′ AGATGAGTCTTGTAAGTCTTGGTAAGAGCCGACCACTTGGTA-3′; 487 bp) and Pax9P-5′GGGTCTCTCTTCTTGTTTGTTGTTGTT-3′; Pax9-5′ATCTAAAACCAAGACTCGGAAGAC-3′; 481 bp).

Primers for qPCR
The primers for qPCR were as follows: 
Gapdh-F: AACCTTGCCATTTGGAAGG, R: GGCCCATCCACAATGTTC; Col2a1-F: GCAGAAGACCAGTTCCACATA, R: CTTGCCCCACCTAGTC; Pax1-F: CCTGGAGGCCAGACATATATATAC, R: GTATACCTGGCTGGTTGGA; Pax9-F: GCAGTGAAAGGATGGAAGAG, R: GATGCTGAGCAGACAATCTGCT; Sox9-F: ACAGACTCACATCTCTCTCTATGCT, R: CTGAGATTGCACAGTGT; Sox5-F: AGAAACCGCTGACTGGGAGTA, R: GATGGGGATGCTGGTTG; Wwp2-F: ATCTATTGCGGACACCAAGAG, R: CCGTGACAAACATCGAG; Acan-F: CTGCCTCTCACGTGAAAAG, R: ACCAGGGCGCTAATGCGTAG.

Histology sectioned in situ hybridization (SISH) and immunohistochemistry and imaging
Mouse embryos processing methods for histology, SISH and immunohistochemistry were performed as described (Chen et al., 1996; Kraus and Lufkin, 1999; Wang et al., 2000; Zhao et al., 2003). Antibodies and dilutions are as follows: Pax1: SC-25407X, 1:200; Pax9: SC-25410X, 1:200; GFP: SC-9996, 1:50; bovine anti-rabbit IgG-B: SC-2363, 1:400 and horse anti-mouse IgG at recommended dilution by the Vectastain® ABC kit (Vector Laboratories; cat # PK-4002). A LEICA M205 FA microscope was used for fluorescence imaging of embryos and sections were imaged using a Zeiss Axio Imager Z1.

Competing interests
The authors declare no competing or financial interests.

Author contributions
V.S., P.K. and T.L. were involved in the design of the study. All the authors were involved in data interpretation. V.S. performed all the experiments, analyses and wrote the manuscript. P.K. carried out all the animal work, SISH and mouse microinjections. V.S., W.S., X.H. and S.P. performed the analyses of ChiP-Seq data and performed data analysis. V.S. and S.L.L. performed ChiP experiments. All authors contributed to the drafting of the manuscript and have read and approved the final manuscript.

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Data availability
The datasets generated during and/or analyzed during the current study are available in GEO (https://www.ncbi.nlm.nih.gov/geo/) under accession numbers: GSE63017 (Pax1, Pax9 microarray), GSE33173 (Sox5/Sox6 microarray) and SRA: SRP046294 (Pax9 ChiP-seq). All other relevant data are provided in the Supplementary Materials.

References


互补问题：这个文档中提到的“Mouse embryos processing methods for histology, SISH and immunohistochemistry were performed as described (Chen et al., 1996; Kraus and Lufkin, 1999; Wang et al., 2000; Zhao et al., 2003).”这句话是否正确？如果是，请解释为什么。如果是，这句话的正确性在哪里？

回答：这个问题的答案是“是的，这句话是正确的。”

解释：这句话是正确的，因为它准确地引用了原始文献。原句中提到的“Mouse embryos processing methods for histology, SISH and immunohistochemistry were performed as described (Chen et al., 1996; Kraus and Lufkin, 1999; Wang et al., 2000; Zhao et al., 2003).”这句话与原始文献中提到的实验方法一致。通过引用这些文献，作者确认了他们使用的实验方法是正确的，并且这些方法已经被其他研究人员验证过。这样做的好处是可以提高论文的可靠性，并且让读者可以参考这些文献来了解更详细的信息。
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