Pax9 is required for cardiovascular development and interacts with Tbx1 in the pharyngeal endoderm to control 4th pharyngeal arch artery morphogenesis.

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AIK, SC: performed RNA-seq data analysis
KS, MN, RM, RK, HP: created the Pax9Cre mouse
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Summary statement
Pax9 is required for outflow tract and aortic arch development, and functions together with Tbx1 in the pharyngeal endoderm for 4th arch artery formation.
Abstract

Developmental defects affecting the heart and aortic arch arteries are a significant phenotype observed in 22q11 deletion syndrome patients and are caused by a microdeletion on chromosome 22q11. *TBX1*, one of the deleted genes, is expressed throughout the pharyngeal arches and is considered a key gene, when mutated, for the arch artery defects. *Pax9* is expressed in the pharyngeal endoderm and is downregulated in *Tbx1* mutant mice. We show here that *Pax9* deficient mice are born with complex cardiovascular malformations affecting the outflow tract and aortic arch arteries with failure of the 3rd and 4th pharyngeal arch arteries to form correctly. Transcriptome analysis indicated that *Pax9* and *Tbx1* may function together, and mice double heterozygous for *Tbx1/Pax9* presented with a significantly increased incidence of interrupted aortic arch when compared to *Tbx1* heterozygous mice. Using a novel *Pax9Cre* allele we demonstrated that the site of this *Tbx1-Pax9* genetic interaction is in the pharyngeal endoderm, therefore revealing that a *Tbx1-Pax9*-controlled signalling mechanism emanating from the pharyngeal endoderm is required for critical tissue interactions during normal morphogenesis of the pharyngeal arch artery system.
Introduction

Conotruncal heart malformations, affecting the outflow tract and aortic arch arteries, occur in 30% of all cases of congenital heart defects (Thom et al., 2006). Approximately 20% of foetuses identified with conotruncal defects by ultrasound are diagnosed with 22q11 deletion syndrome (22q11DS) (Boudjemline et al., 2001), the most common microdeletion syndrome with an incidence of 1:4000 live births (Scambler, 2000). Patients typically have a 3Mb deletion on chromosome 22 that encompasses 45 protein coding genes (Morrow et al., 2018) and ~80% of patients present with some form of congenital cardiovascular defect (Momma, 2010), of which one of the most common observed is interruption of the aortic arch (IAA) (Unolt et al., 2018). Although clinically rare in the general population, approximately 50% of all cases of IAA occur in 22q11DS patients (Boudjemline et al., 2001; Lewin et al., 1997; Van Mierop and Kutsche, 1986). IAA is a consequence of the left 4th pharyngeal arch artery (PAA) failing to form correctly during embryonic development. There are five pairs of PAAs that arise within a series of repeated protuberances on either side of the developing pharynx known as the pharyngeal arches. These arches consist of paraxial mesoderm- and neural crest cell (NCC)-derived mesenchyme, and are externally enclosed by ectoderm and internally lined by endoderm (Graham, 2003). The initially bilaterally symmetrical PAAs develop sequentially in a cranial to caudal sequence during embryogenesis but undergo a complex remodelling process that is conserved in mammals to form the asymmetrical mature aortic arch configuration (Bamforth et al., 2013; Hiruma et al., 2002). Of the genes deleted in 22q11DS, hemizygosity of \textit{TBX1} is considered to be the cause of the cardiovascular defects, with point mutations in \textit{TBX1} also identified in patients with 22q11DS-like phenotypes (Yagi et al., 2003; Zweier et al., 2007). \textit{Tbx1} is expressed throughout the pharyngeal arches within the ectoderm, endoderm and mesoderm cells and is critical for pharyngeal arch and PAA development (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Complete loss of \textit{Tbx1} from the mouse results in a failure of the pharyngeal arches to form correctly resulting in hypoplasia of the second arch and aplasia of the remaining caudal arches, leading to an absent thymus and common arterial trunk (Jerome and Papaioannou, 2001; Merscher et al., 2001). In contrast, heterozygous deletion of \textit{Tbx1} predominantly affects development of the 4th PAA resulting in IAA in a minority of mice with aberrant right-subclavian artery (A-RSA) observed more frequently (Lindsay et al., 2001). \textit{Tbx1} heterozygotes may, however, also present with VSD, overriding aorta, double aortic arch and an abnormal thymus (Merscher et al., 2001; Zhang and Baldini, 2008). Although almost all 22q11DS patients are hemizygous for the deletion, the phenotypic spectrum is highly variable (Unolt et al., 2018), thus it has been proposed that genes outside of the deleted region may impact on the clinical phenotype (Guo et al.,...
Several genes have been identified as potential modifiers of 22q11DS as determined by genetic interaction studies and transcriptome analyses in Tbx1 mutant mice (Aggarwal and Morrow, 2008; Ivins et al., 2005; Liao et al., 2008; Papangeli and Scambler, 2013). One such candidate gene is Pax9 whose expression in the pharyngeal endoderm was found to be significantly reduced in Tbx1-deficient embryos (Ivins et al., 2005; Liao et al., 2008). Pax9 belongs to a family of Pax genes encoding for transcription factors involved in various cellular roles during embryonic development (Chi and Epstein, 2002; Mansouri et al., 1996) and is specifically expressed in the endoderm of all four pharyngeal pouches of the mouse by E9.5 (Neubuser et al., 1995). At later stages in mouse development, Pax9 is expressed in the oesophagus, somites and limbs, and within the neural crest contributing to craniofacial development (Peters et al., 1998). Embryos deficient for Pax9 exhibit craniofacial defects, including cleft secondary palate as well as absent teeth, skeletal defects, and lack derivatives of the 3rd and 4th pharyngeal pouches, i.e. the thymus, parathyroids and ultimobranchial bodies (Peters et al., 1998) yet the role of Pax9 in cardiovascular development has not been determined.

In this study we have investigated cardiovascular development in Pax9-deficient mice, and show that loss of Pax9 leads to complex cardiovascular defects affecting the outflow tract and aortic arch arteries. We also uncover a strong genetic interaction between Tbx1 and Pax9 that leads to 4th PAA-derived defects in double heterozygous mice, and this interaction is cell-autonomous within the pharyngeal endoderm.

**Results**

**Pax9-deficient embryos have cardiovascular defects.**

Mice lacking Pax9 die perinatally and exhibit craniofacial, pharyngeal gland and skeletal defects (Peters et al., 1998). As the cause of death was unclear we carefully re-analysed Pax9−/− neonates from a Pax9+/- intercross and found that all Pax9−/− neonates had a patterning defect of their aortic arch arteries which included an IAA with A-RSA (Fig. 1B; Table 1). To investigate these defects in more detail we processed Pax9−/− embryos for histology and magnetic resonance imaging (MRI) and found that Pax9−/− embryos presented with a wide range of cardiovascular abnormalities (Fig. 1C-Q; Table 1). These defects included double outlet right ventricle with interventricular communication (Fig. 1F, J, K), IAA and A-RSA (Fig. 1G, K). The common carotid arteries were also frequently absent resulting
in unilateral or bilateral internal and external carotids arising directly from the dorsal aorta and the A-RSA (Fig. 1H, J-L). *Pax9*−/− embryos also had a hypoplastic aorta (Fig. 1N-O) and bicuspid aortic valves (Fig. 1Q).

Developmental defects of the arch arteries occur when the PAAs fail to form or remodel correctly. To investigate the morphogenesis of the PAAs we performed high-resolution episcopic microscopy (HREM) on E10.5 and E11.5 *Pax9*−/− embryos and manually segmented the datasets to create 3-D images of the PAA. Embryos were stage-matched by somite counting to exclude developmental delay as a cause of observed defects. Analysis of E10.5 coronal sections revealed that the 3rd and 4th pharyngeal arches of *Pax9*−/− embryos were smaller compared to *Pax9*+/− controls, as previously reported (Peters et al., 1998), but did show segmentation to individual arches (Fig. 2A-C). At E10.5 the developing PAAs are bilaterally symmetrical, having formed in a cranial to caudal sequence. The 1st and 2nd PAAs have normally regressed by this stage, and the 3rd, 4th and 6th PAAs are present (Bamforth et al., 2013; Hiruma et al., 2002) (Fig. 2A). In *Pax9*−/− embryos the 1st and 2nd PAAs were abnormally persistent, the 3rd PAAs were either absent, hypoplastic or interrupted, and the 4th PAAs were absent (Fig. 2B, C; Table 2). The aortic sac was also hypoplastic in *Pax9*−/− embryos compared to the controls (Fig. 2B, C). Intra-cardiac injection of India ink at E10.5-E11.0 showed that, in controls, the caudal PAAs were patent to ink and of equivalent size (Fig. 2D). In *Pax9*−/− embryos, however, persisting 1st and 2nd PAAs were patent to ink, and the 3rd PAAs were hypoplastic (Fig. 2J-L) or non-patent to ink, and therefore presumed absent. The majority of the 4th PAAs were bilaterally non-patent to ink (Fig. 2J-L; Table 2). Immunohistochemical staining for Pecam1 showed that the endothelium within the 3rd and 4th pharyngeal arches had formed lumenised PAAs at E10.5 in control embryos (Fig. 2G), but in *Pax9*−/− embryos the 3rd PAAs were visibly smaller in size and only isolated endothelial cells were seen within the 4th pharyngeal arch (Fig. 2H, I).

During E11.5 of mouse development, the PAA system begins to remodel and becomes asymmetric in appearance (Bamforth et al., 2013; Hiruma et al., 2002). In *Pax9* control embryos at this stage the right 6th PAAs appeared thinner and the distal outflow tract was septated. The carotid duct, the region of the dorsal aorta between the 3rd and 4th PAAs, had begun to involute but the 3rd, 4th and 6th PAAs remained connected to the dorsal aortae (Fig. 3A). In *Pax9*−/− embryos at E11.5, evidence of presumed persisting 1st or 2nd PAAs were observed extending anteriorly from the aortic sac, and the 3rd PAAs were also affected, either unilaterally or bilaterally, and were either absent, hypoplastic or interrupted and not connected to the dorsal aortae (Fig. 3B-D). The carotid ducts were maintained, and this, coupled with the abnormal detachment of the 3rd PAAs from the dorsal aorta and the
persistence of the 1st or 2nd PAAs, resulted in an absent common carotid artery and the internal and external carotid arteries arising directly from the aortic arch arteries as observed at E15.5 (Fig. 1). Additionally, the distal outflow tract was unseptated and the 4th PAAs were bilaterally absent (Fig. 3B-D; Table 2).

Looping of the heart tube at E10.5 was not affected in Pax9<sup>−/−</sup> embryos (Fig. 3E, F). In normal mouse development the outflow tract cushions rotate through an anticlockwise direction (Bajolle et al., 2006) resulting in the major septal and parietal cushions being positioned side by side (Fig. 3G-I). In Pax9<sup>−/−</sup> embryos, however, by the end of the E11.5 stage these cushions were aberrantly positioned, with the parietal cushion more anterior to the septal cushion, indicating that a clockwise rotation of the outflow tract cushions had occurred (Fig. 3J-L).

Pharyngeal endoderm signalling influences neural crest cell differentiation.

During normal embryogenesis, a subset of NCC, the cardiac neural crest, migrate into the caudal pharyngeal arches to provide support to the developing PAAs and also contribute to outflow tract septation (Hutson and Kirby, 2003). Pax9 is predominantly expressed in the pharyngeal endoderm (Fig. S1), but is also known to be expressed within the NCC contributing to craniofacial development (Kist et al., 2007), although expression in cardiac NCC has not been described. We therefore looked to see if a cell autonomous loss of Pax9 from NCC resulted in cardiovascular defects. Conditional deletion of Pax9 using Wnt1Cre transgenic mice (Danielian et al., 1998), and the Pax9<sup>lox</sup> allele (Kist et al., 2007), however, did not result in cardiovascular abnormalities, although cleft palate was observed as expected (Fig. 4A-D). We next looked to see if the migration of NCC was affected in global Pax9<sup>−/−</sup> embryos using Wnt1Cre and eYFP (Srinivas et al., 2001) reporter mice to create embryos where the NCC could be lineage traced. Live fluorescence imaging of Pax9<sup>+/−</sup>;Wnt1Cre;eYFP embryos at E9.5 and E10.5 revealed NCC present in the head and dorsal root ganglia, as well as migrating towards and filling the pharyngeal arches (Fig. 4E, G). A similar pattern was observed in Pax9<sup>−/−</sup>;Wnt1Cre;eYFP embryos (Fig. 4F, H). Counting of NCC revealed no significant change in their number in the 3<sup>rd</sup> pharyngeal arch at E9.5, but a significant reduction was found in the 3<sup>rd</sup> and 4<sup>th</sup> arches at E10.5 (Fig. 4K).

Immunostaining for BrdU incorporation specifically in NCC showed no significant difference in NCC proliferation in the caudal pharyngeal arches (Fig. 4L-N), and very little apoptosis was seen in both control and Pax9<sup>−/−</sup> embryos (Fig. 4O-Q). These observations indicated that the migration of NCC per se to the pharyngeal arches were not affected in Pax9<sup>−/−</sup>
embryos. Given that NCC differentiate into the smooth muscle cells (SMC) that support the remodelling PAAs (Waldo et al., 1996), we looked to see if SMC recruitment to the 3rd PAAs was affected in Pax9<sup>−/−</sup> embryos. Sections from E10.5 and E11.5 embryos were immunostained using an anti-alpha smooth muscle actin antibody to visualise SMC around the PAAs. Evidence of SMC recruitment to the 3rd PAAs was seen in each control embryo examined (Fig. 4R-U), however very little SMC investment around the 3rd PAAs in Pax9<sup>−/−</sup> embryo sections was observed (Fig. 4V-Y). These results indicated that the 3rd PAAs in Pax9<sup>−/−</sup> embryos form but collapse from a failure of SMC investment.

**Tbx1 and Pax9 interact in 4th PAA formation**

To further investigate the role of Pax9 in cardiovascular development, we looked at the effect of Pax9 loss on the pharyngeal arch transcriptome at E9.5 (Fig. 5A-C). Analysis of the RNA-seq data identified 3863 significant differentially expressed genes (Table S1). Gene set enrichment analysis revealed several significantly down-regulated pathways including “extracellular matrix organisation” and “axon guidance” (Fig. 5D). Interestingly, Tbx1 was significantly reduced in Pax9<sup>−/−</sup> embryos at E9.5, and this was confirmed by qPCR (Fig. 5I). We also confirmed that Pax9 expression was reduced in Tbx1-null embryos (Fig. S2). As Tbx1 expression is crucial for 4th PAA morphogenesis (Lindsay et al., 2001; Zhang et al., 2005) these data suggested that Pax9 may function together with Tbx1 rather than being downstream of it. We therefore compiled a list of all the genes reported to be significantly differentially expressed in Tbx1-null embryos from published microarray (Ivins et al., 2005; Liao et al., 2008; van Bueren et al., 2010), and mouse genetic interaction studies, giving 1476 unique Tbx1-related genes. These were then compared to all genes significantly changed in Pax9<sup>−/−</sup> embryo pharyngeal arch tissue giving 342 genes that were common to both datasets (Fig. 5E-G; Fig. S3; Table S1) and suggesting that Tbx1 and Pax9 may share a genetic network. Among these common genes were Chd7 and Gbx2, which have been shown to interact with Tbx1 in mice (Calmont et al., 2009; Randall et al., 2009), and were also reduced in Pax9-null tissue by qPCR and in situ hybridisation (Fig. 5H-S).

To investigate a potential genetic interaction in vivo between Tbx1 and Pax9 we confirmed that both genes were co-expressed in the pharyngeal endoderm (Fig. 6A-G). We then crossed Tbx1<sup>+/-</sup> mice (Jerome and Papaioannou, 2001) with Pax9<sup>−/−</sup> mice to create Tbx1<sup>+/-</sup>;Pax9<sup>−/−</sup> double heterozygotes. Genotype analysis of 168 pups at weaning revealed that only two Tbx1<sup>+/-</sup>;Pax9<sup>−/−</sup> mice were alive, a highly significant deviation from the 42 expected of each genotype from this cross (p=1.4x10<sup>-12</sup>; Table S2). To identify the timing of lethality, neonates were observed from the day of birth with all Tbx1<sup>+/-</sup>;Pax9<sup>−/−</sup> mutants dying...
during the first 24 h. Examination of neonatal aortic arch arteries revealed that all controls were normal (Fig. 6H) while Tbx1<sup>-/-</sup>;Pax9<sup>-/-</sup> neonates frequently presented with A-RSA (Fig. 6I; Table 1). All dead Tbx1<sup>-/-</sup>;Pax9<sup>-/-</sup> neonates had 4<sup>th</sup> PAA-derived defects including IAA (Fig. 6J, K; Table 1).

Having established postnatal cardiovascular defects we next analysed E15.5 Tbx1<sup>-/-</sup>;Pax9<sup>-/-</sup> embryos by MRI (Fig. 6L-O). All wild-type and Pax9<sup>-/-</sup> embryos were normal. Of the Tbx1<sup>-/-</sup> embryos examined, a high proportion had some form of 4<sup>th</sup> PAA-derived defect, including IAA (Fig. 6M; Table 1). All Tbx1<sup>-/-</sup>;Pax9<sup>-/-</sup> embryos examined, however, had some form of 4<sup>th</sup> PAA-derived defect (Fig. 6N, O; Table 1) with the incidence of IAA being significantly increased compared to Tbx1<sup>-/-</sup> embryos (p<0.001). The incidence of perimembranous VSD was also significantly increased (p=0.04), but none of the embryos examined displayed outflow tract defects or bicuspid aortic valve, although an abnormal thymus was frequently observed (Fig. S4; Table S3). Tbx1<sup>-/-</sup>;Pax9<sup>-/-</sup> embryos at E10.5 were injected intra-cardially with ink (Fig. 6P-S) and the patency of the developing 4<sup>th</sup> PAAs was then compared between Tbx1<sup>-/-</sup> and Tbx1<sup>-/-</sup>;Pax9<sup>-/-</sup> embryos (Table 2). All Tbx1<sup>-/-</sup> embryos displayed a predominantly hypoplastic 4<sup>th</sup> PAA defect, with 50% of embryos having a unilateral, and 50% a bilateral, defect. In Tbx1<sup>-/-</sup>;Pax9<sup>-/-</sup> embryos a significant increase in bilateral aplasia of the 4<sup>th</sup> PAAs (p=0.004) was found, but no defects of PAAs 1, 2, 3 or 6 were seen. RNA analysis showed that Tbx1, Pax9, Chd7 and Gbx2 RNA levels were not significantly changed between the respective single heterozygotes and Tbx1<sup>-/-</sup>;Pax9<sup>-/-</sup> pharyngeal arches (Fig. 6T-W).

**Tbx1 and Pax9 interact cell autonomously in the pharyngeal endoderm for 4<sup>th</sup> PAA formation**

The presumed site of the Tbx1-Pax9 interaction is the pharyngeal endoderm, and to demonstrate a cell-autonomous interaction in this tissue we crossed Tbx1<sup>flo</sup> mice (Xu et al., 2004), with a novel Pax9Cre transgenic mouse (Fig. 7A-C). This Pax9Cre strain has the Cre recombinase gene inserted into the Pax9 coding region and effectively results in a Pax9-null allele. The Pax9Cre allele functions as expected by activating R26R<sup>lacZ</sup> expression specifically in the pharyngeal endoderm from E9.5 to E11.5 (Fig. 7D-G; Fig. S5) and Pax9Cre;Pax9<sup>flo</sup> neonates (i.e. Pax9-null) die at birth with the typical Pax9<sup>-/-</sup> phenotype (Fig. 7H-Q). To test the specificity of the Pax9Cre allele the pharyngeal arches of Pax9Cre;eYFP E9.5 embryos (Fig. 8A) were dissociated into single cells and flow-sorted into eYFP-positive and -negative populations. The eYFP-positive population was significantly enriched for eYFP and Pax9 transcripts (Fig. 8B, C). Tbx1<sup>flo</sup> and Pax9Cre mice were
intercrossed to create Tbx1+/flo;Pax9Cre embryos which are double heterozygous for each gene but only in the Pax9 expression domain. A significant reduction in Tbx1 RNA levels was observed in eYFP-positive flow-sorted pharyngeal endoderm cells from conditionally deleted E9.5 mutant embryos compared to Pax9Cre;eYFP controls (Fig. 8D). Litters from a Tbx1+/flo and Pax9Cre cross were collected on the day of birth, and a significant reduction in the expected number of Tbx1+/flo;Pax9Cre mutants was found (p=2.4x10^{-5}; Table S4) and 62.5% of these mutants displayed an arch artery defect (Fig. 8F; Table 1). We then collected embryos (E13.5-E15.5) and analysed them by MRI or µCT which revealed that 38.5% of mutants presented with an arch artery defect (Fig. 8H; Table 1) and an abnormal thymus (Fig. S4; Table S3). We also collected embryos at E10.5 and injected them intracardially with ink to visualise the PAAs. All Tbx1+/flo control embryos analysed had normal PAAs, with all three vessels being patent to ink and of a similar diameter, whilst the majority of Tbx1+/flo;Pax9Cre mutant embryos presented with some form of 4th PAA defect (Fig. 8J; Table 2).

In summary, our data show that Pax9 is critical for cardiovascular development, and when lost the PAAs and outflow tract fail to develop correctly resulting in postnatal death. The 4th PAAs do not form and the 3rd PAAs collapse with reduced investment from vascular SMC. Our data also demonstrate that Tbx1 and associated genes are deregulated in Pax9-null embryos, and Tbx1 and Pax9 function together in the pharyngeal endoderm for 4th PAA morphogenesis.

Discussion

Loss of Pax9 results in complex cardiovascular defects

Pax9 plays a key role in cardiovascular development as Pax9⁻/⁻ neonates and embryos display a range of cardiovascular defects. Gene mutations and chromosomal deletions that include PAX9 have been identified in patients with cardiovascular abnormalities, and other conditions (Table S5), suggesting that PAX9 has potential importance in human cardiovascular development. Of these, one patient with a 105 kb hemizygous deletion of 14q13 presented with IAA, a hypoplastic aorta, bicuspid aortic valve, and a ventricular septal defect (Santen et al., 2012), all of which appear with high penetrance in mouse Pax9-null embryos. This small deletion only removed PAX9, NKX2-1 and NKX2-8, although deletion of
either Nkx2 gene in mice does not result in any cardiovascular defects (Kimura et al., 1996; Pabst et al., 2003). It is therefore possible that hemizygous deletion of PAX9 in humans can recapitulate, in part, the mouse knockout phenotype.

**Tbx1 and Pax9 interact in 4th PAA development**

Published microarray studies investigating the transcriptome of Tbx1-null embryos have implicated Pax9 as a downstream target of Tbx1 (Ivins et al., 2005; Liao et al., 2008). Further evidence of a cross-talk between Tbx1 and Pax9 has been suggested via the interaction of Ripply3 (Okubo et al., 2011). This protein, which is a Tbx1 repressor, was shown to prevent Tbx1 activation of luciferase from a Pax9 promoter construct, and Pax9 expression in the pharyngeal endoderm was increased in Ripply3-null embryos suggesting that Tbx1 may be upstream of Pax9. Ripply3 levels were not significantly changed in our Pax9-null pharyngeal arch transcriptome data, but Tbx1 levels were significantly reduced indicating that perhaps Pax9 and Tbx1 may function together rather than work in a hierarchal pathway. This is further supported by analysis of Tbx1 and Pax9 RNA levels in Tbx1\(^{+/−}\);Pax9\(^{+/−}\) embryos, which were not significantly changed compared to levels seen in the single heterozygotes. Further analysis identified that many genes differentially expressed in Tbx1-null embryos, or shown to interact with Tbx1 in mouse models, were also significantly differentially expressed in Pax9-null embryos, e.g. Chd7 and Gbx2. Mice heterozygous for Chd7 have abnormal 4th PAA derivatives and a combined heterozygosity with Tbx1 results in an increased incidence of 4th PAA defects (Randall et al., 2009). Gbx2-null embryos display cardiovascular defects (Byrd and Meyers, 2005) and Tbx1 and Gbx2 have also been shown to genetically interact in PAA development (Calmont et al., 2009). Although both these Tbx1 targets have been demonstrated to interact in the pharyngeal ectoderm, they are also expressed in the pharyngeal endoderm and could therefore interact with Pax9 either directly or indirectly in this tissue. As our data strongly suggested that Pax9 may interact with Tbx1 and its related genes in the morphogenesis of the PAAs we therefore looked for a genetic interaction between Tbx1 and Pax9 in mice. We found that all mice examined (embryos and neonates) double heterozygous for each gene (i.e. Tbx1\(^{+/−}\);Pax9\(^{+/−}\)) displayed some defect associated with abnormal development of the 4th PAA, most notably IAA-B. Although hypoplasia or absence of the 4th PAAs is a feature in mouse embryos heterozygous for Tbx1 (Lindsay et al., 2001), this phenotype was significantly more prevalent in Tbx1\(^{+/−}\);Pax9\(^{+/−}\) mice, and much higher than observed in Tbx1\(^{+/−}\) embryos. It therefore appears that combining one Pax9-null allele with one Tbx1-null allele exacerbates the Tbx1\(^{+/−}\) phenotype.
We used our novel Pax9Cre mouse to conditionally delete Tbx1 from the pharyngeal endoderm, whilst simultaneously creating double haploinsufficiency for the two genes in this tissue. A highly significant reduction in the number of Tbx1+/flox;Pax9Cre neonates born was observed indicating that heterozygous loss of Tbx1 and Pax9 from the pharyngeal endoderm has a major impact on post-natal survival, with almost two-thirds of the recovered neonates examined having identifiable cardiovascular defects. A similar proportion of affected mutants was found at foetal stages. A vascular ring structure was observed in Tbx1+/flox;Pax9Cre mutants (Fig. 8H), similar to that described in Lgdel/+ mice, which are chromosomally engineered to be hemizygous for Tbx1 (Merscher et al., 2001), and also in Six1;Eya1 mutant embryos (Guo et al., 2011a). Eya1 was significantly reduced in Pax9-null pharyngeal arch tissue. When embryos were examined at mid-embryogenesis, however, we found that almost all displayed 4th PAA defects. When compared to our Tbx1+/−;Pax9+/− embryo data it was apparent that there was no significant difference in the incidence of bilateral 4th PAA defects between Tbx1+/flox;Pax9Cre and Tbx1+/−;Pax9−/− embryos. This indicates that the conditional heterozygous deletion of Tbx1 from the pharyngeal endoderm, in the context of Pax9 heterozygosity, has the same effect on 4th PAA morphogenesis as seen in the global Tbx1+/−;Pax9−/− embryos at E10.5. Although the ink injection data from Tbx1+/flox;Pax9Cre embryos matches that seen in Tbx1+/−;Pax9−/− embryos, and shows that heterozygous loss of Tbx1 from the pharyngeal endoderm severely affects PAA morphogenesis, there is a discrepancy in the cardiovascular phenotype observed at foetal and neonatal stages that does not reflect that seen at mid-embryogenesis, nor that observed in Tbx1+/−;Pax9−/− embryos. It is well documented, however, that the Tbx1+/− 4th PAA phenotype does recover during development (see Table S6). It therefore appears that a conditional deletion of Tbx1 from the pharyngeal endoderm in the context of Pax9 haploinsufficiency replicates the Tbx1 heterozygous arch artery phenotype in terms of a highly penetrant 4th PAA defect at mid-embryogenesis that partially recovers by the foetal stage. Our data, however, does raise the question as to whether we are seeing the effect of a pharyngeal endoderm specific loss of Tbx1 with Pax9Cre, or a genetic interaction occurring between Tbx1 and Pax9 in the pharyngeal endoderm. Conditional deletion experiments have been performed in transgenic mice to identify the role of Tbx1 in specific tissues and have examined the effect of Tbx1 heterozygosity in the pharyngeal mesoderm and/or epithelium for 4th PAA development (see Table S7). When compared, our data clearly shows that the 4th PAA phenotype is much more penetrant than that seen in embryos only heterozygous for Tbx1 in the pharyngeal epithelia. We therefore conclude that we are showing a strong genetic and cell-autonomous interaction between Tbx1 and Pax9 within the pharyngeal endoderm which impacts on 4th PAA morphogenesis. Moreover, and in support of a phenotype extending beyond what is expected from a Tbx1 heterozygous phenotype, our Tbx1flox;Pax9Cre mice display a high
incidence of perinatal lethality, a feature not previously described in $Tbx1^{+/−}$ mice. Interestingly, the homozygous deletion of $Tbx1$ from the pharyngeal endoderm using Sox17Cre mice resulted in pharyngeal arch aplasia but not common arterial trunk, although IAA was observed (Jackson et al., 2014). This phenotype is similar to that seen in our $Tbx1;Pax9$ mutants but is distinct from either $Tbx1$ and $Pax9$ constitutive null mutants.

Pax9 signalling from the pharyngeal endoderm is required for 3rd PAA maintenance

The mammalian PAAs develop symmetrically in a cranial to caudal sequence, and then subsequently remodel to form the typical asymmetric aortic arch artery system seen in the adult (Bamforth et al., 2013; Hiruma et al., 2002). In normal PAA development the 1st artery forms first, followed by the 2nd, but these two vessels rapidly remodel and contribute to capillary beds and the mandibular and hyoid arteries. In some $Pax9^{−/−}$ embryos, these vessels aberrantly persisted into E10.5 and E11.5 and may contribute to the anomalous arrangement of the aortic arch arteries when the 3rd PAAs collapse. In normal development the 3rd PAAs will form part of the common and proximal internal carotid arteries, which elongate as the embryo grows. The carotid duct involutes and the dorsal aorta anterior to this segment will form the distal internal carotid artery, with the external carotid artery derived from the proximal parts of the 1st and 2nd PAA (Hiruma et al., 2002). The persistence of the 1st and/or 2nd PAAs in $Pax9^{−/−}$ embryos, coupled with the failure of the 3rd PAAs to be maintained and the carotid duct to involute, results in the external carotid arteries rising directly from the aorta, and the internal carotid arteries from the anterior dorsal aortae. This phenomenon has been described clinically (Roberts and Gerald, 1978). Although reduced numbers of NCC were found in the caudal pharyngeal arches at E10.5, migration, apoptosis or proliferation of NCC did not appear to be impaired in $Pax9$-null embryos. It is well recognised that NCC differentiate into the SMC that are important for the stabilisation of the remodelled PAAs (Hutson and Kirby, 2003) and it is likely that the failure of SMC to envelop the 3rd PAAs in $Pax9$-null embryos results in the collapse of this vessel. This has also been described in $Hoxa3$-null embryos (Kameda, 2009) and reduced NCC migration in genetic mouse and surgical chick models also leads to defects of the 3rd PAAs (Bradshaw et al., 2009; Epstein et al., 2000; Franz, 1989; Nishibatake et al., 1987). We were unable to look for a similar occurrence in the 4th PAAs as this fails to form in the majority of $Pax9$-null embryos. Signalling between the pharyngeal endoderm and NCC does occur (Graham et al., 2005) so it is therefore possible that signals emanating from the pharyngeal endoderm under Pax9 control are necessary to influence the differentiation of NCC into SMC, and without these signals the 3rd PAAs are not supported during the remodelling phase of arch artery
development and they collapse. The top two GSEA pathways identified from the RNA-seq data were “extracellular matrix organisation” and “axon guidance”, and these could be relevant for a NCC derived phenotype particularly as the extracellular matrix is known to be crucial for the migration of NCC (Henderson and Copp, 1997; Perris and Perissinotto, 2000) and the slit, ephrin, semaphorin and plexin genes listed under “axon guidance” are usually associated with NCC expression or migration in cardiovascular development (Epstein et al., 2015; Kirby and Hutson, 2010; Sun et al., 2018). There is, however, an alternative explanation for the collapse of the 3rd PAAs. Haemodynamic force plays a major role in artery development (Culver and Dickinson, 2010; Yashiro et al., 2007), so it is possible that the 3rd PAA defect is a consequence of increased blood flow to the persistent 1st/2nd PAAs which prevents sufficient blood flow to the 3rd PAAs, and flow-induced arteriogenesis is impaired. This may also be an explanation for the failure of the 4th PAAs to form. A reduced haemodynamic force would also explain the reduction in SMC recruitment as this is also dependent on blood flow (Padget et al., 2019). Although endothelial cells were present within the 4th pharyngeal arch of Pax9-null embryos they apparently failed to condense into tubes, a feature that has also been described in both Tbx1-null and Gbx2-null embryos (Byrd and Meyers, 2005; Calmont et al., 2009).

The double outlet right ventricle phenotype observed at E15.5 in Pax9-null embryos probably arises from the marked outflow tract rotation defect due to a change in the aortic to pulmonary valve axis angle (Bostrom and Hutchins, 1988; Lomonico et al., 1986) which is also observed in Pax3 and Pitx2c mutant hearts (Bajolle et al., 2006).

In summary, Pax9 expression in the pharyngeal endoderm has regional (anterior/posterior) roles in regulating the morphogenesis of the PAAs (Fig. 9). In the 3rd pharyngeal arch Pax9 is important for controlling NCC differentiation into the SMC that support the remodelling vessel, whereas in the 4th pharyngeal arch endoderm, the interaction of Pax9 with Tbx1 and its targets is crucial for the formation of the 4th PAAs.
Materials and methods

Mice
The mice used in this study have previously been described: Pax9<sup>+/−</sup> (Peters et al., 1998), Pax9<sup>fl</sup> (Kist et al., 2007), Tbx<sup>+/−</sup> (Jerome and Papaioannou, 2001), Tbx1<sup>fl</sup> (Xu et al., 2004), Wnt1Cre (Danielian et al., 1998), R26<sup>Cre</sup> (Srinivas et al., 2001), R26<sup>lacZ</sup> (Soriano, 1999) and FLPe (Dymecki, 1996). All mice were maintained on a C57Bl/6J genetic background. All studies involving animals were performed in accordance with UK Home Office Animals (Scientific Procedures) Act 1986.

Generation of Pax9Cre mice
The Pax9Cre targeting construct was designed to replace a 1.06 kb section of the Pax9 gene, spanning the second half of exon two, containing the start codon, and the first half of the 3<sup>rd</sup> exon, containing the paired box and octapeptide motif, with a promoterless Cre recombinase gene preceded by a consensus Kozak sequence, in the pPNT4 targeting vector (Conrad et al., 2003), using standard molecular biology methods (Fig. 7A). The 1.3kb right homology arm, generated as previously described (Kist et al., 2005), was inserted between the FRT-flanked PGK-Neomycin cassette and the HSV-Thymidine Kinase cassette of pPNT4. The unwanted loxP site of pPNT4 was removed by XbaI partial digestion, followed by Klenow treatment and re-ligation. The left homology arm was generated as a 7.4 kb Sall to Smal fragment of the Pax9 genomic region including exon 1 and the first half of the second exon, and inserted 5' of the Cre recombinase gene. The targeting vector was linearized with SfiI and mouse ES cells were targeted and selected as previously described (Kearns et al., 2013). Southern blot analysis was performed according to standard methods following XbaI digest of genomic DNA. An external southern probe was used to verify integration of the targeting cassette in the 5’ region (Fig. 7B) and PCR was used to validate integration of the 3’ region (not shown). The FRT-flanked PGK-Neomycin cassette was removed by crossing the derived Pax9Cre-Neo mice with FLPe mice (Dymecki, 1996) to create Pax9Cre mice, which were then backcrossed to strain C57Bl/6J for >6 generations. Genotyping by multiplex PCR [primers: F1-<sup>ACTCAAGCCTCTTTCC</sup> (common), R1-<sup>TGTCTCATGTGCTGCCGC</sup> (Pax9 exon 2), and R2-<sup>GTTGGACGACGCTTGTC</sup> (Cre)] were used to simultaneously identify the wild-type and Pax9Cre alleles (Fig. 7C).
Breeding
Male and female mice were mated and the detection of a vaginal plug the next morning considered to be E0.5. Pregnant females were culled on the required day and embryos collected. Embryos at E9.5-E11.5 were staged by somite counting.

Imaging
Magnetic resonance imaging (MRI) was performed using a 9.4T MR system (Varian, US) as previously described (Bamforth et al., 2012; Schneider et al., 2004) on embryos at E15.5. High Resolution Episcopic Microscopy (HREM) and micro-computed tomography (µCT) techniques have been described in detail elsewhere (Degenhardt et al., 2010; Geyer et al., 2009). All MRI, HREM and µCT images were converted into a volume data set and segmented using Amira software (ThermoFisher Scientific) to create 2- and 3-dimensional (3-D) images. Structures were manually outlined using the label field function of Amira and surface rendered to produce the 3-D images. Intra-cardiac ink injections were performed as described (Calmont et al., 2009). Aorta measurements were taken from the region just proximal to the point at which the ascending aorta becomes the aortic arch. Haematoxylin and eosin and X-gal staining, and Pax9 whole-mount in situ hybridisation, were performed using standard techniques. mRNA expression was examined by in situ hybridisation using RNAscope ® Multiplex Fluorescent v2 Assay (Advanced Cell Diagnostics, Newark, CA, USA) following the manufacturer’s instructions. Probe details given in Table S8. For proliferation assays BrdU (Sigma) dissolved in PBS was administered by intraperitoneal injection at a dose of 50mg/kg to pregnant dams one hour before embryo collection. Immunohistochemistry was performed using standard techniques (antibody details in Table S8) on paraformaldehyde fixed embryo sections, and fluorescently imaged on an Axioimager (Zeiss). To assess an apoptotic index within the pharyngeal arches, control and Pax9−/− embryos at E9.5 (n=3 per genotype; 24-28 somites) and E10.5 (n=3 per genotype; 30-35 somites) were examined following immunostaining with the caspase-3 antibody. To assess a proliferative index within the NCC in the pharyngeal arches, control and Pax9−/− embryos expressing Wnt1Cre activated eYFP at E9.5 (n=3 per genotype; 23-28 somites) and E10.5 (n≥4 for each genotype; 31-39 somites) were examined following immunostaining with anti-BrdU and anti-GFP antibodies. All positively stained and DAPI positive cells within the pharyngeal arches were counted and the ratio of positively stained cells over the total number of cells calculated. The average number of NCC within the caudal pharyngeal arches was calculated at E9.5 (n=3 per genotype, 23-28 somites) and E10.5 (n=4 per genotype, 32-34 somites). For all cell counting experiments at least three sections per embryo were assessed.
Transcriptome analysis

Wild-type (Pax9\textsuperscript{+/+}) and Pax9-null (Pax9\textsuperscript{−/−}) embryos at E9.5 (n=3 of each genotype, stage-matched for 26-27 somites) were collected in ice-cold DEPC-PBS, and the pharyngeal region, from the junction between the first and second pharyngeal arch to the base of the heart, dissected free in RNA\textit{later} (Sigma). Genotypes were identified by PCR from the embryo tails and/or yolk sac material. Total RNA was extracted using the QIAGEN Plus Micro kit with genomic DNA Eliminator columns, and total RNA eluted in 30 μl RNase-free water. RNA concentration and purity were assessed by NanoDrop spectrophotometry and Agilent Bioanalyzer with all samples producing a RIN of 10. Genomic DNA contamination was excluded by PCR for intronic regions (sensitivity 5 pg/μl). To confirm that the correct genotypes were processed, cDNA was made from 100 ng total RNA and a qPCR for Pax9 performed (Fig. 5B). The total RNA samples were shipped on dry-ice to EMBLM-GeneCore (Heidelberg, Germany) for RNA-seq first-strand specific library preparation using Illumina TruSeq, and 100bp paired end reads were generated from an Illumina HiSeq 2000 Sequencer. Quality control by FastQC suggested a small amount of adaptor contamination in each sample, so raw reads were trimmed of adaptor sequence with Trimmomatic (Bolger et al., 2014). Reads shorter than 36bp after trimming were discarded, and reads whose pair had been discarded were also removed. Trimmed reads were further filtered to keep only uniquely aligned and non-redundant reads to remove a vast majority of pseudogenes caused by multi-mapping. Also, to avoid bias due to imbalanced library sizes among conditions, all samples were sub-sampled towards the smallest sample (mt-663_1). The resulting reads were aligned to the Mouse Genome (Ensembl release 81, GRCm38) using the splice aware read aligner STAR 2 (star-2.5.2a-0, 20/01/2017) (Dobin et al., 2013). Finally, aligned reads were assigned to Ensembl genes using featureCounts (Liao et al., 2014) with default parameters. These count tables were imported into R and analysed using the package LIMMA (Ritchie et al., 2015) and voom function from Bioconductor (Gentleman et al., 2004). A gene set enrichment analysis tool (Subramanian et al., 2005) was used to predict signalling pathways that were affected when gene expression in the wild-type and the Pax9 mutant samples was compared. All resulting pathways were filtered for a familywise-error rate (p<0.001) to correct for multiple hypotheses testing.
Data accessibility
The RNA-seq data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE128087 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128087).

Flow cytometry
The pharyngeal arch region of E9.5 Pax9Cre;eYFP control (n=5; 23-27 somites) and mutant Tbx1^{+/flox};Pax9Cre;eYFP (n=5; 24-29 somites) embryos was dissected free as described above and dissociated to single cells with Accumax (eBioscience) by incubating at 37°C for 30 minutes. The reaction was stopped by the addition of 10% fetal calf serum (FCS), the cells washed in PBS, and resuspended in 10% FCS. Cells were stained with propidium iodide. Fluorescence activated cell sorting was performed on a Becton Dickinson FACS Aria II using a 100μm nozzle and a sheath pressure of 20 psi. Single cells were gated using FSC-A vs SSC-A followed by FSC-A vs FSC-H and FSC-A vs SSC-W to remove any doublets. Live single cells were gated using propidium iodide vs FSC-A, and this population finally gated on eYFP-positive and -negative cells and sorted into collection tubes.

Quantitative real-time RT-PCR (qPCR)
RNA, from either dissociated pharyngeal arch cells or flow-sorted cells, was extracted using Trizol reagent (Invitrogen) combined with a Purelink RNA Mini Kit (Ambion) and on-column DNase1 treatment. RNA was eluted with 30 µl RNase-free water. Total RNA was converted to cDNA using a High-Capacity cDNA Reverse transcription kit (Applied Biosystems) and random hexamers. qPCR was performed using SYBR Green JumpStart Taq ReadyMix (Sigma) using the primers listed in Table S9. All qPCR reactions were performed in triplicate on a QuantStudio 7 Real-Time PCR System (Thermo Fisher). Data were analysed using the comparative Ct method (Schmittgen and Livak, 2008).

Statistical analysis
Chi-squared test was used to compare genotype frequencies of litters, and Pearson's chi-squared test for associations was used to compare defect frequencies between the different genotypes. For analysis of qPCR data, aorta measurements, and cell counts, datasets were tested for variance using the Shapiro-Wilk test (Prism 8.01 software, GraphPad). Two-tailed unpaired t-tests were performed on normally distributed data, and a two-tailed Mann-Whitney U non-parametric test was used for not normally distributed data. One-way ANOVA with Tukey's multiple comparison test was used to analyse qPCR data from four embryo genotypes. A hypergeometric distribution test was used to calculate a p value for observing an overlap between two gene lists in R. Groups were considered significantly different when
\( p < 0.05, \text{ or } p \leq 0.1 \) for RNA-seq data. No statistical methods were used to predetermine sample size which were chosen based on previous experience to obtain statistical significance and reproducibility. No data points were excluded, and all data collected from each individual experiment were used for analysis.

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**Competing interests**

No competing interests declared.

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References


Figure 1. Loss of Pax9 results in cardiovascular developmental defects.

A, Arch arteries of control neonates were normal (n=8). B, Pax9<sup>–/–</sup> neonates (n=5) displayed IAA-B, absent right subclavian artery (presumed retro-esophageal), and atypical right and left carotid arteries (LC, RC). Scale, 1mm. C-E, Pax9<sup>+/–</sup> embryos at E14.5 show normal outflow tract, arch arteries and thymus. F-H, In Pax9<sup>–/–</sup> embryos (n=4) the aorta (Ao) arises aberrantly from the right ventricle giving double outlet right ventricle (DORV; F). Hypoplastic aorta, IAA-B, and an A-RSA are present (G). More cranially the thymus is absent and aberrant internal and external carotid arteries (iRCA, eRCA, iLCA, eLCA) are seen. I-L, 3-D reconstructions of E15.5 hearts from MRI datasets. I, Pax9<sup>+/+</sup> embryo with normal aortic arch.
arteries. **J-L**, In Pax9<sup>−/−</sup> embryos (n=15) defects seen are DORV with interventricular communication (IVC), IAA (type B or C), A-RSA (retro-esophageal in J, K and isolated in L) and aberrant carotid arteries. Scale, 500μm. **M-O**, The ascending aorta is significantly smaller in Pax9<sup>−/−</sup> embryos (n=5) compared to Pax9<sup>+/−</sup> control embryos (n=7). Mean ± s.e.m.; ****<i>p</i>&lt;0.0001; two-tailed unpaired t-test. **P**, Pax9<sup>−/−</sup> control embryos have three normal aortic valve leaflets (n=6), the right (RC), left (LC) and non-coronary (NC). **Q**, Pax9<sup>−/−</sup> embryos (n=5) have bicuspid aortic valve. Scale, 100μm. Abbreviations: Ao, aorta; AoA, aortic arch; AD, arterial duct; BC, brachiocephalic; dAo, dorsal aorta; LCC, left common carotid artery; LSA, left subclavian artery; LV, left ventricle; RCC, right common carotid artery; RSA, right subclavian artery; RV, right ventricle; Th, thymus.
**Figure 2.** Pax9 loss causes aberrations in pharyngeal arch artery formation.

A-C, Coronal views of control (Pax9+/+) and mutant (Pax9−/) embryos at E10.5 (30-40s), examined by high-resolution episcopic microscopy. A, In Pax9+/+ control embryos (n=5) the 3rd, 4th and 6th PAAs are of equal size and bilaterally symmetrical. B, C, In Pax9−/− embryos (n=4) the 4th PAAs were bilaterally absent (*asterisk*), the 3rd PAAs and aortic sac (as) were hypoplastic, and the 1st and/or 2nd PAAs abnormally persisted. D-F, Intracardiac ink injection into E10.5-E11.0 (31-42s) embryos. D, In control embryos (n=12) PAAs 3-6 are patent to ink, are of equivalent diameter and bilaterally symmetrical. E, F, In Pax9−/− embryos (n=12) the 3rd PAAs are hypoplastic and the 4th PAAs non-patent to ink (*asterisk*). F, The 1st and 2nd PAAs are persisting anomalously. G-I, Immunostaining using Pecam1 antibody at E10.5 (31-36s). G, Control embryos (n=3) have a ring of Pecam1 labelled endothelium lining the 3rd and 4th PAAs. H, I, In Pax9−/− embryos (n=5) the 3rd PAAs are visibly smaller and disorganized endothelial cells are within the 4th pharyngeal arch (pa; *arrows*). Somite counts (s) indicated. Scale, 100μm.
Figure 3. *Pax9*−/− embryos display aberrant rotation of the outflow tract and abnormal PAA remodelling.

Image datasets were acquired by high-resolution episcopic microscopy. A-D, 3-D reconstructions of the PAAs at E11.5 (45-50s). A, In *Pax9*+/* control embryos (n=5) the outflow tract is septated, the right 6th PAA has thinned and the carotid duct (cd) has begun to involute. B-D, In *Pax9*−/* embryos (n=4) the 4th PAAs are absent and the 3rd PAAs are aberrantly connected or detached from the dorsal aorta (*asterisks*). The carotid duct has failed to involute and septation of the outflow tract is delayed. Persisting 1st or 2nd PAAs are observed coursing anteriorly from the abnormal 3rd PAAs (B, D) or from the aortic sac (C). E, F, Looping of the heart tube at E10.5 was not affected in *Pax9*−/* embryos (n=4). G-L, Outflow tract rotation from E10.5 to E11.5 (39-49s). G-I, In control embryos the major outflow tract cushions (parietal in *green*; septal in *yellow*) rotated in an anti-clockwise direction resulting in the cushions being aligned side-by-side. J-L, In *Pax9*−/* embryos the cushions rotated in a clockwise direction resulting in the parietal cushion anterior to the septal cushion. Abbreviations: as, aortic sac; at, aortic trunk; da, dorsal aorta; pa, pulmonary arteries; pt, pulmonary trunk. Somite (s) counts indicated. Scale, 100μm.
Figure 4. Failure in smooth muscle cell recruitment causes the 3rd PAAs to collapse.

A-D, E15.5 embryos with a Pax9 conditional deletion in neural crest cells (NCC) were examined by MRI. A, Control embryos had a normal palate (P). B, Pax9+/−;Wnt1Cre mutant embryos (n=6) had cleft palate (CP). C, D, No cardiovascular defects were observed in control or mutant embryos. Abbreviations as in Figure 1. E-J, NCC in Pax9+/+ (E, G, I) or Pax9−/− (F, H, J) embryos were labelled using Wnt1Cre and eYFP alleles. E, F, Fluorescence imaging showed no difference in NCC migration into the pharyngeal arches.
(arrowheads) at E9.5 (n≥3, 23-25s) and E10.5 (n≥5, 32-36s). I, J, Fluorescent embryos were sectioned coronally and immunostained using anti-GFP and anti-Pax9 antibodies. K, NCC were counted from immunostained sections. *p<0.05, ***p<0.001; two-tailed unpaired t-test. L-M, Embryo sections were immunostained with anti-BrdU and anti-GFP antibodies to detect proliferation in NCC in Pax9+/+ (L) and Pax9−/− (M) embryos. N, No significant difference in the rate of proliferation was found between control and Pax9−/− NCC in the caudal arches at E9.5 (n=3, 23-28s) or E10.5 (n≥4, 31-39s). O-P, Embryo sections immunostained with an anti-Caspase3 antibody to detect apoptosis in the caudal pharyngeal arches of Pax9+/+ (N) and Pax9−/− (O) embryos Q, No significant difference in the rate of apoptosis was found between control and Pax9−/− embryos at E9.5 (n=3, 24-28s) or E10.5 (n=3, 30-35s). Two-tailed unpaired t-test. R-Y, Embryo sections were immunostained using anti-αSMA for smooth muscle and anti-Pecam1 (R, V) or ERG (T, U, X, Y) antibodies for endothelium or anti-GFP to label NCC in Wnt1Cre;eYFP embryos (S, W). In all control embryos SMC surrounded the 3rd PAAs (E10.5, n=7, 31-36s, R, S; and E11.5, n=3, 43-45s, T, U; red arrowhead). In Pax9−/− embryos the 3rd PAAs had limited recruitment of SMC (E10.5, n=6; 32-35s, V, W; and E11.5, n=3, 42-45s, X, Y; white arrowhead). Abbreviations: da, dorsal aorta; en, endoderm; mc, mesenchyme; ns, not significant; pa, pharyngeal arch. Somite counts (s) indicated. Scales: A - D, 500μm; E-O, 100μm; R-Y, 50μm.
Figure 5. Pax9 shares a genetic network with Tbx1.

A, The pharyngeal arch region of control and Pax9<sup>−/−</sup> embryos at E9.5 (n=3 per genotype; 26-27s) was dissected (indicated by dashed lines). Scale, 500µm. B, Total RNA was extracted, converted to cDNA and checked by qPCR. C, Principle component analysis of RNA-seq data showed a clear separation between control and mutant samples (shown on PC2) and only slight biological variation across replicates (shown on PC1). D,
Gene set enrichment analysis identified signalling pathways predicted to be significantly down-regulated in Pax9\textsuperscript{−/−} embryos. All pathways shown have a familywise-error rate of \( p<0.001 \). E, Tbx1-related genes (n=1476) were compared to those genes significantly differentially expressed (DE) in Pax9\textsuperscript{−/−} embryo tissue (n=3863, adjusted \( p \) value \( \leq 0.1 \)) giving 342 genes common to both datasets (*\( p=0.0029 \), as assessed by a hypergeometric distribution test). Examples of genes in each cohort are shown. F, Volcano plot highlighting Tbx1 interacting genes significantly DE in Pax9\textsuperscript{−/−} embryos (based on adjusted \( p \) value \( \leq 0.1 \)). G, Heat map showing colour-coded (red=up, blue=down) expression levels of DE genes between Pax9\textsuperscript{+/+} (wt) and Pax9\textsuperscript{−/−} (mt) embryos that are common to Tbx1 DE genes (adjusted \( p \) value \( \leq 0.05 \) are shown). H-K, qPCR analysis confirmed that Pax9 (H), Tbx1 (I), Chd7 (J) and Gbx2 (K) were significantly reduced in Pax9\textsuperscript{−/−} pharyngeal tissue at E9.5 (n=5 embryos per genotype). **\( p<0.01 \); two-tailed unpaired t-test (Tbx1 and Chd7) and two-tailed Mann-Whitney (Pax9 and Gbx2). L-S, RNAscope \textit{in situ} hybridisation showed a reduced expression of Pax9 (M), Tbx1 (O), Chd7 (Q) and Gbx2 (S) in the pharyngeal endoderm in Pax9\textsuperscript{−/−} embryos at E9.5 (n=3 per genotype, 23-28s). Abbreviations: en, endoderm; me, mesoderm; pa, pharyngeal arch. Somite counts (s) indicated. Scale, 50µm.
Figure 6. Tbx1;Pax9 double heterozygous embryos have 4th PAA defects.

Tbx1 and Pax9 expression in the pharyngeal arches was examined at E9.5. A-D, RNAscope in situ hybridisation expression of Tbx1 in the 2nd and 3rd pharyngeal arch (pa) endoderm (en) and mesoderm (me; A) and Pax9 expression in the endoderm only (B). The signals overlap in the endoderm (C, and higher power in C'). D, DAPI staining. E-G, Immunostaining for Tbx1 and Pax9 (E) demonstrates protein co-localisation within cells of the pharyngeal endoderm. Single staining for Tbx1 (F) and Pax9 (G) is shown. H-K, Neonates from a Tbx1+/− and Pax9+/− intercross were recovered and the aortic arches examined. H, Arch
arteries were normal in all Pax9<sup>−/−</sup> neonates (n=10). I, Tbx1<sup>−/−</sup> neonates (n=6) often presented with A-RSA, inferred by the absence of the brachiocephalic and right subclavian arteries. J, K, All Tbx1<sup>−/−</sup>; Pax9<sup>−/−</sup> neonates (n=9) died within 24 h of birth with IAA and/or A-RSA. L-O, 3-D reconstructions of E15.5 embryo hearts from MRI datasets. L, Pax9<sup>−/−</sup> hearts were normal (n=15). M, Tbx1<sup>−/−</sup> hearts (n=19) frequently displayed 4<sup>th</sup> PAA defects such as A-RSA. N, O, All Tbx1<sup>−/−</sup>; Pax9<sup>−/−</sup> embryos (n=20) presented with a 4<sup>th</sup> PAA-derived defect such as IAA-B, A-RSA and/or cervical right subclavian artery (cRSA). P-S, Intracardiac ink injections into E10.5 embryos (27-38s). P, PAAs in control embryos were normal (n=10). Q, In Tbx1<sup>−/−</sup> embryos (n=8) the 4<sup>th</sup> PAAs were often hypoplastic. R, S, In Tbx1<sup>−/−</sup>;Pax9<sup>−/−</sup> embryos (n=9) the 4<sup>th</sup> PAAs were frequently bilaterally absent. Abbreviations as Figure 1. Somite counts (s) indicated. Scale: H-K, 2mm; L-O, 500μm; A-D, P-S, 100μm, E-G, 50μm. T-W, qPCR analysis in wild-type (WT), Tbx1<sup>−/−</sup>, Pax9<sup>−/−</sup> and Tbx1<sup>−/−</sup>;Pax9<sup>−/−</sup> embryos (n≥3 per genotype, somite range 23-27). T, Tbx1 levels were significantly reduced in Tbx1<sup>−/−</sup> and Tbx1<sup>−/−</sup>;Pax9<sup>−/−</sup> embryos. U, Pax9 levels were significantly reduced in Pax9<sup>−/−</sup> and Tbx1<sup>−/−</sup>;Pax9<sup>−/−</sup> embryos. There was no significant difference in Pax9 (T), Tbx1 (U), Chd7 (V) or Gbx2 (W) levels between single heterozygotes and Tbx1<sup>−/−</sup>;Pax9<sup>−/−</sup> embryos. One-way ANOVA with Tukey's multiple comparison test. ns, not significant; *p<0.05; **p<0.01; ****p<0.0001.
Figure 7. Generation of the Pax9Cre mouse.

A, A Pax9Cre targeting vector was created with a Cre cassette inserted into the Smal restriction sites found in exons 2 and 3 of the Pax9 gene. For transformation the vector was linearised with Sfil. B, Correctly targeted ES cells were identified by Southern blotting on XbaI (X) digested genomic DNA. The derived Pax9Cre-Neo mice were crossed with FLPe-expressing mice to remove the Neomycin cassette and create Pax9Cre mice, which were genotyped using the indicated primers (C). D-G, Pax9Cre mice were mated with R26RlacZ.
reporter mice and embryos were stained with X-Gal at E8.5 (n=3), E9.5 (n=7), E10.5 (n=6) and E11.5 (n=5). Positive staining in regions known to express Pax9 was observed (alb, anterior limb bud; np, nasal process; pp, pharyngeal pouch; vc, vertebral column). H-Q, Pax9Cre and Pax9flox/flox mice were crossed to create Pax9+/flox (control) and Pax9Cre/flox (mutant) mice. All Pax9Cre/flox neonates died on the day of birth and presented with the typical Pax9-null phenotype (n=5), including a bloated abdomen (I, arrow) and pre-axial digit duplication (d) in the hind limb (K, arrow). Sections of E14.5-E15.5 embryos (n=6) showed cleft palate (CP; M), absent thymus and cardiovascular defects (O, Q). Abbreviations as Figure 1. Somite counts (s) indicated. Scale, 500µm.
**Figure 8.** Conditional deletion of *Tbx1* from the pharyngeal endoderm.

*A*, *Pax9Cre* drives expression of the eYFP reporter gene in the pharyngeal endoderm at E9.5. Scale, 500 µm. *B*-*D*, Flow sorting and qPCR of eYFP-positive and -negative pharyngeal arch cells from *Pax9Cre*;eYFP control and *Tbx1*+/flo*;Pax9Cre* E9.5 embryos (*n*=5 of each; dissected as shown in *A*). From control embryos the eYFP-positive cells (*yellow circles*) were significantly enriched for eYFP (*B*) and *Pax9* (*C*) compared to eYFP-negative cells (*black squares*). *D*, There was a significant reduction in *Tbx1* levels in *Tbx1*+/flo*;Pax9Cre* (mutant) eYFP-positive cells (*yellow squares*) compared to controls (*yellow circles*). *p*<0.05, **p**<0.01; two-tailed unpaired t-test. *E*-*J*, Cardiovascular defects in *Tbx1*+/flo*;Pax9Cre* mice. *E*, *F*, Neonates were collected shortly after birth and the aortic arch arteries examined. *E*, Control neonates (*n*=39 wild-type, *n*=43 *Pax9Cre* and *n*=33 *Tbx1*+/flo*) all had normal arch arteries. *F*, *Tbx1*+/flo*;Pax9Cre* mutants (*n*=8) had cardiovascular defects such as a right-sided aorta (R-Ao) and A-RSA. Scale, 1mm. *G*, *H*, Embryos at E13.5-E15.5 were analysed by imaging (*n*=26 mutants). *G*, Control embryo with normal cardiovascular system. *H*, In the mutant there is a vascular ring formed by a R-Ao and an A-RSA. Scale, 500µm. *I*, *J*, Embryos collected at E10.5 (38-43s) were injected intra-cardially with ink. *I*, In
Tbx1*/* flox control embryos (n=15) PAAs 3-6 were patent to ink. J, In Tbx1*/* flox;Pax9Cre mutant embryos (n=17) the 4th PAA was frequently absent. Somite counts (s) are indicated. Scale, 100µm. Abbreviations as Figure 1.
Figure 9. Proposed model for role of Pax9 mediated signalling during pharyngeal arch artery development.

A, In wild-type embryos Pax9 expression in the 3rd pharyngeal arch endoderm induces neural crest cells (NCC), either directly or indirectly, to differentiate into the SMC that invest around the endothelium of the remodelling 3rd PAAs. In the 4th arch endoderm, Tbx1 and Pax9 function together to, most likely indirectly, control formation of the 4th PAAs, potentially through collaboration with Chd7 and Gbx2. B, In Pax9 deficient embryos, signals from the 3rd pharyngeal arch endoderm are compromised and NCC differentiation into SMC is impaired resulting in the collapse of the 3rd PAAs by E11.5. In Tbx1-Pax9 double heterozygous embryos, reduced levels of Tbx1 and Pax9 in the 4th pharyngeal arch endoderm are insufficient to allow the 4th PAAs to form.
### Table 1. Cardiovascular defects observed in mutant embryos (>E13.5) and neonates.

Pax9<sup>−/−</sup> neonates (n=5) were analysed by dissection, embryos at E14.5 by histology (n=4) and at E15.5 by MRI (n=15). Tbx1<sup>+/−</sup> (n=19) and Tbx1<sup>+/−</sup>;Pax9<sup>−/−</sup> (n=20) embryos were analysed by MRI. Tbx1<sup>+/−</sup>;Pax9Cre embryos were analysed by MRI (n=17) and µCT (n=9). All wild-type (n=16), Pax9<sup>+/−</sup> (n=15) and Tbx1<sup>+/−</sup>;Pax9<sup>lox/lox</sup> (n=5) control embryos assessed were normal. There was a statistically significant increase in the incidence of VSD and IAA in the Tbx1<sup>+/−</sup>;Pax9<sup>−/−</sup> embryos compared to the Tbx1<sup>+/−</sup> embryos. *p<0.05; **p<0.0001; Pearson's chi-squared test for associations.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stage</th>
<th>n</th>
<th>VSD</th>
<th>DORV + IVC</th>
<th>IAA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IAA + A-RSA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A-RSA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Vascular ring&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cervical AoA + A-RSA</th>
<th>Absent CC&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax9&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Neonate</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
<td>5 (100%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (40%)</td>
</tr>
<tr>
<td></td>
<td>E14.5 - E15.5</td>
<td>19</td>
<td>3</td>
<td>15</td>
<td>3</td>
<td>14 (74%)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>15 (79%)</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Neonate</td>
<td>6</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
<td>1 (17%)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/−&lt;/sup&gt;;Pax9&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>E15.5</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (5%)</td>
<td>9</td>
<td>0</td>
<td>2 (11%)</td>
<td>0</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/−&lt;/sup&gt;;Pax9&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>E13.5 - E15.5</td>
<td>20</td>
<td>6*</td>
<td>0</td>
<td>0</td>
<td>14** (70%)</td>
<td>3</td>
<td>0</td>
<td>3 (15%)</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Combined incidence of IAA, irrespective of co-occurrence with A-RSA, in Pax9<sup>−/−</sup> neonates and embryos (n=24), was 92% (n=19 type-B and n=3 type-C). In Tbx1 mutants IAA was always type-B.

<sup>b</sup> A-RSA refers to a retroesophageal, cervical origin or isolated right subclavian artery. Two Pax9<sup>−/−</sup> embryos had isolated RSA.

<sup>c</sup> Vascular ring is used to describe a right-sided aorta with A-RSA.
Absent common carotid artery (CC), resulting in the internal and external carotid arteries arising directly from the main aortic vessels, unilaterally (n=4) or bilaterally (n=11) in Pax9−/− embryos.

Abbreviations: AoA, aortic arch; DORV + IVC, double outlet right ventricle with interventricular communication; VSD, perimembranous ventricular septal defect; NA, not assessed.
Table 2. Pharyngeal arch artery defects.

Embryos were collected and assessed for PAA defects. Pax9<–/– embryos were either injected intra-cardially with ink (E10.5; n=12) or imaged using HREM (E10.5-E11.5; n=8) to visualise the PAA. Data are combined. For Pax9<–/– embryos each left and right PAA 1-4 was scored as having a unilateral or bilateral defect, and the bilateral defects categorised as either present, a combination of hypoplastic, interrupted and/or absent (Hypo/Int/Abs), and bilaterally absent. For Tbx1;Pax9 genotypes ink injection was performed at E10.5 and only the 4th PAA was scored. The increase in bilaterally absent 4th PAA in Tbx1<+/-;<Pax9<+/- embryos is significant compared to Tbx1<+/- embryos; *p=0.004; Pearson’s chi-squared test for associations. All control embryos, Pax9<+/- (n=18), Pax9<+/- (n=25), were normal.
Figure S1. Pax9 expression during mouse pharyngeal development. A – C, coronal, and D – F, transverse sections of embryos at E9.5 (A), E10.5 (B), E11.5 (C, D), E12.5 (E) and E15.5 (F). Pharyngeal arch arteries (paa) are outlined. Pax9 is expressed in the pharyngeal endoderm at E9.5 and E10.5, and is subsequently expressed within the esophagus (es), developing thymus (th) and ultimobranchial bodies (ub) of the pharyngeal region. Somite counts (s) indicated. Abbreviations: L/R-4th, left/right 4th pharyngeal arch artery; lcc, left common carotid; oft, outflow tract; pa, pharyngeal arch; pp, pharyngeal pouch; ph, pharynx; rcc, right common carotid; tr, trachea. Scale, 100μm.
Figure S2. Pax9 mRNA is reduced in the pharyngeal region of Tbx1⁻/⁻ embryos. Whole-mount in situ hybridization using a Pax9 riboprobe demonstrates that Pax9 is specifically expressed in the pharyngeal endoderm and somites at E9.5 in wild-type (A) and Tbx1⁺/- (B) embryos. C, In Tbx1⁻/⁻ embryos, however, Pax9 levels are dramatically reduced from the pharyngeal endoderm (arrow). Somite counts (s) indicated. Scale: A – C, 500μm; A’ – C’, 100μm.
Figure S3. Relationship between shared genes differentially expressed in Tbx1-null and Pax9-null datasets. Shared genes (n=329) between the Tbx1-null and Pax9-null datasets were compared to see if they were concordant (i.e. genes went up in both datasets, or down), or if they were discordant (i.e. went up in one dataset but down in the other). This revealed that 35% of shared genes were both down, but also 63% of shared genes were down in Tbx1-nulls and up in Pax9-nulls. Examples of genes in each category are shown in the Table.

<table>
<thead>
<tr>
<th>Gene up or down per dataset</th>
<th>Concordant</th>
<th>Discordant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tbx1 up, Pax9 up</strong></td>
<td><strong>Tbx1 down, Pax9 down</strong></td>
<td><strong>Tbx1 up, Pax9 down</strong></td>
</tr>
<tr>
<td>n</td>
<td>2</td>
<td>114</td>
</tr>
<tr>
<td>Examples of genes in each category</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisd2, CxCl13</td>
<td>Aldh1a1, Gbx2, Gli3, Myh9, Notch3, Pax9, Pbx1, Tbx1</td>
<td>Col3a1, Col6a1, Kif1b, Sparcl1, Vtn</td>
</tr>
</tbody>
</table>

Figure S3. Relationship between shared genes differentially expressed in Tbx1-null and Pax9-null datasets. Shared genes (n=329) between the Tbx1-null and Pax9-null datasets were compared to see if they were concordant (i.e. genes went up in both datasets, or down), or if they were discordant (i.e. went up in one dataset but down in the other). This revealed that 35% of shared genes were both down, but also 63% of shared genes were down in Tbx1-nulls and up in Pax9-nulls. Examples of genes in each category are shown in the Table.
Figure S4. Thymus abnormalities are seen in Tbx1;Pax9 mutant embryos. An abnormally placed thymus was frequently observed in conjunction with arch artery defects. A, Normally placed thymic lobes (purple) in a wild-type control embryo. The thymic lobes are placed ventrally to the aortic arch arteries. B, C, In a Tbx1+/–;Pax9+/– mutant embryo (B) and a Tbx1+/–;Pax9Cre mutant embryo (C), both with interrupted aortic arch and aberrant right subclavian artery (A-RSA), the thymic lobes are split and asymmetrically placed. The right and left common carotid arteries can be seen traversing between the two thymic lobes. Abbreviations: LCC, left common carotid; LSA, left subclavian artery; RCC, right common carotid; Th, thymus. Scale, 500µm.

Figure S5. Reporter gene expression from the Pax9Cre allele. A, B, Cre mediated recombination of lacZ by Pax9Cre induces expression in the pharyngeal endoderm at E9.5. Scale, 500 µm.
Supplementary Tables

Table S1 (Excel file). Differentially expressed genes \((p \leq 0.1)\) from RNA-seq analysis of Pax9-null pharyngeal arch tissue. **Sheet A, B,** The top up- and down-regulated coding genes from the Pax9 RNA-seq data are listed and ranked by log2 fold-change, with a cut off -0.59 for the down-regulated genes (**A**; \(n=647\)) and 0.59 for the up-regulated genes (**B**; \(n=240\)). **Sheet C, D,** Values of the Tbx1-Pax9 shared genes from the Pax9 RNA-seq data, ranked by fold-change. **C,** Genes down-regulated in Pax9-null pharyngeal arch tissue \((n=122)\). **D,** Genes up-regulated in Pax9-null pharyngeal arch tissue \((n=220)\).

Click here to Download Table S1

Table S2. Genotypes of weaned pups (at 3 weeks of age), neonates (collected within 24 h of birth) and embryos (E15.5 and E9.5) from a Tbx1\(^{+/−}\) and Pax9\(^{+/−}\) cross. There was a significant loss of expected pups at weaning (Chi-squared test; **** \(p=1.4\times10^{-12}\)). There was no significant difference from the expected number of pups for each possible genotype in neonates or embryos.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Weaning</th>
<th>Neonate</th>
<th>E15.5</th>
<th>E9.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>55</td>
<td>7</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Pax9(^{+/−})</td>
<td>68</td>
<td>10</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>Tbx1(^{+/−})</td>
<td>43</td>
<td>6</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Tbx1(^{+/−});Pax9(^{+/−})</td>
<td>2 ****</td>
<td>9</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Total</td>
<td>168</td>
<td>32</td>
<td>70</td>
<td>74</td>
</tr>
<tr>
<td>(n) expected for each genotype</td>
<td>42</td>
<td>8.0</td>
<td>17.5</td>
<td>18.5</td>
</tr>
</tbody>
</table>

Table S3. Summary of thymus phenotypes observed in Tbx1 and Tbx1;Pax9 mutant embryos. The thymus was frequently seen to be asymmetric in appearance and split apart in Tbx1\(^{+/−}\);Pax9\(^{+/−}\) and Tbx1\(^{+/−}\);Pax9Cre mutants. An abnormal thymus was always associated with an arch artery defect. One Tbx1\(^{+/−}\);Pax9\(^{+/−}\) mutant presented with an absent thymus and an unusual holoprosencephaly type phenotype (not shown).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stage</th>
<th>(n)</th>
<th>Normal</th>
<th>Split &amp; asymmetric</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbx1(^{+/−})</td>
<td>E15.5</td>
<td>19</td>
<td>15</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(79%)</td>
<td>(21%)</td>
<td></td>
</tr>
<tr>
<td>Tbx1(^{+/−});Pax9(^{+/−})</td>
<td>20</td>
<td>3</td>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(15%)</td>
<td>(80%)</td>
<td>(5%)</td>
</tr>
<tr>
<td>Tbx1(^{+/−});Pax9Cre</td>
<td>E13.5 -E15.5</td>
<td>26</td>
<td>16</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(61.5%)</td>
<td>(38.5%)</td>
<td></td>
</tr>
</tbody>
</table>
Table S4. Genotypes of neonates (collected within 48 h of birth) from a $Tbx1^{+/\text{flo}x} \times \text{Pax9Cre}$ cross. Foetuses (E13.5 – E15.5) and embryos (E10.5) were from a $Tbx1^{\text{flo}x/\text{flo}x} \times \text{Pax9Cre}$ cross. There was a significant loss of expected neonates (Chi-squared test; **** $p=2.4\times10^{-5}$).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Neonate</th>
<th>Foetus</th>
<th>Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>39</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pax9Cre</td>
<td>43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$Tbx1^{+/\text{flo}x}$</td>
<td>33</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>$Tbx1^{+/\text{flo}x}\times\text{Pax9Cre}$</td>
<td>8****</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>123</td>
<td>35</td>
<td>32</td>
</tr>
<tr>
<td>$n$ expected for each genotype</td>
<td>30.75</td>
<td>17.5</td>
<td>16</td>
</tr>
</tbody>
</table>

Table S5. $PAX9$ mutations and aberrant expression levels that have been associated with human disease.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mutation/karyotype</th>
<th>Detail</th>
<th>Other</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congenital Heart Defect</td>
<td>14q13 (105kb deletion)</td>
<td>IAA-B, BAV, hypoplastic aorta, VSD</td>
<td>Facial dysmorphism</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>46,XY,del(14) (11.2q13)</td>
<td>Persistent foramen ovale</td>
<td>Craniofacial defects</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>46,XY,del(14) (11.2q13)</td>
<td>Patent foramen ovale and patent ductus arteriosus</td>
<td>Craniofacial defects, delayed bone ossification</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>14q13.3 (884kb deletion)</td>
<td>Persistent pulmonary hypertension of the newborn</td>
<td>Oligodontia, hypothyroidism</td>
<td>4</td>
</tr>
<tr>
<td>Craniofacial PAX9 mutations</td>
<td></td>
<td>Hypo/oligodontia</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cleft lip/palate</td>
<td>Hypodontia</td>
<td>6,7</td>
</tr>
<tr>
<td>Skeletal</td>
<td>46,XY,t(14;18) (q13;q12)</td>
<td>Mesomelic bone dysplasia</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jarcho-Levin Syndrome</td>
<td>Reduction in PAX9 expression</td>
<td>9</td>
</tr>
<tr>
<td>Cancer</td>
<td>14q13.3</td>
<td>Lung cancer</td>
<td>PAX9 expression amplified</td>
<td>10</td>
</tr>
</tbody>
</table>

$PAX9$ is located at 14q13.3.

Abbreviations: BAV, bicuspid aortic valve; IAA-B, interrupted aortic arch type B; VSD, ventricular septal defect.
Table S6. Penetrance of 4th PAA defects in Tbx1<sup>+/−</sup> and Tbx1<sup>+/flox;Pax9Cre</sup> mutant embryos assessed from published and our data.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stage</th>
<th>n</th>
<th>% defects&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbx1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>E10.5–E11.0</td>
<td>127</td>
<td>87% (63-100%)</td>
<td>11-16 &amp; this study</td>
</tr>
<tr>
<td></td>
<td>E11.5</td>
<td>76</td>
<td>62% (50-74%)</td>
<td>15-17</td>
</tr>
<tr>
<td></td>
<td>Fetal (E14.5–P2)</td>
<td>215</td>
<td>34% (18-49%)</td>
<td>11-15,17-19 &amp; this study</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/flox;Pax9Cre&lt;/sup&gt;</td>
<td>E10.5</td>
<td>17</td>
<td>92%</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Fetal (E13.5–E15.5)</td>
<td>18</td>
<td>33%</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup> For Tbx1<sup>+/−</sup> mice, % defects are given as the mean incidence of abnormalities for the studies cited, with 95% confidence intervals.

Table S7. Penetrance of 4th PAA defects following heterozygous deletion of Tbx1 (globally or conditionally) from E10.5 embryos and assessed by intra-cardiac ink injection or histology, from published and our data.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tissue affected</th>
<th>n</th>
<th>Abnormal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tbx1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Endoderm, mesoderm, ectoderm</td>
<td>127</td>
<td>87%</td>
<td>11-16 &amp; this study</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/−;Pax9&lt;sup&gt;+/−&lt;/sup&gt;&lt;/sup&gt;</td>
<td>Endoderm, mesoderm, ectoderm</td>
<td>9</td>
<td>100%</td>
<td>This study</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/flox;Pax9Cre&lt;/sup&gt;</td>
<td>Endoderm</td>
<td>17</td>
<td>92%</td>
<td>This study</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/flox;Foxg1Cre&lt;/sup&gt;</td>
<td>Endoderm*</td>
<td>30</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/flox;Mesp1Cre&lt;/sup&gt;</td>
<td>Mesoderm</td>
<td>19</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/flox;Foxg1Cre&lt;/sup&gt;</td>
<td>Endoderm, mesoderm, ectoderm</td>
<td>21</td>
<td>42%</td>
<td>21</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/flox;Fgf15Cre&lt;/sup&gt;</td>
<td>Endoderm, ectoderm</td>
<td>34</td>
<td>20%</td>
<td>21</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/flox;Hoxa3Cre&lt;/sup&gt;</td>
<td>Endoderm, mesoderm, ectoderm (neural crest)</td>
<td>17</td>
<td>50%</td>
<td>21</td>
</tr>
<tr>
<td>Tbx1&lt;sup&gt;+/flox;AP2aCre&lt;/sup&gt;</td>
<td>Ectoderm (neural crest)</td>
<td>9*</td>
<td>0</td>
<td>13</td>
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* Mice were maintained congenic in the Swiss Webster background to restrict Cre expression to the pharyngeal endoderm.  
** Tbx1<sup>+/flox;AP2aCre</sup> phenotype assessed at E15.5 by histology.  
N.B. Tbx1 is not expressed in neural crest cells.
### Table S8. Antibodies and probes used for immunostaining and in situ hybridisation.

<table>
<thead>
<tr>
<th>Target</th>
<th>Catalogue number</th>
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<th>Dilution</th>
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<td><em>Primary antibodies</em></td>
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<td>Rabbit polyclonal</td>
<td></td>
<td>1:100</td>
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### Table S9. qPCR primer sequences

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*Development: doi:10.1242/dev.177618: Supplementary information*
References


