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Endoderm-specific deletion of *Tbx1* reveals an FGF-independent role for *Tbx1* in pharyngeal apparatus morphogenesis

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Abstract

Background—The T-box transcription factor *Tbx1*, is essential for the normal development of multiple organ systems in the embryo. One of the most striking phenotypes in *Tbx1*^{−/−} embryos is the failure of the caudal pharyngeal pouches to evaginate from the foregut endoderm. Despite considerable interest in the role of *Tbx1* in development, the mechanisms whereby *Tbx1* controls caudal pouch formation have remained elusive. In particular, the question as to how *Tbx1* expression in the pharyngeal endoderm regulates pharyngeal pouch morphogenesis in the mouse embryo is not known.

Results—To address this question, we produced mouse embryos in which *Tbx1* was specifically deleted from the pharyngeal endoderm and as expected, embryos failed to form caudal pharyngeal pouches. To determine the molecular mechanism, we examined expression of *Fgf3* and *Fgf8* ligands and downstream effectors. Although *Fgf8* expression is greatly reduced in *Tbx1*-deficient endoderm, FGF signaling levels are unaffected. Furthermore, pouch morphogenesis is only partially perturbed by the loss of both *Fgf3* and *Fgf8* from the endoderm, indicating that neither are required for pouch formation.

Conclusions—*Tbx1* deletion from the pharyngeal endoderm is sufficient to cause caudal pharyngeal arch segmentation defects by FGF-independent effectors that remain to be identified.

Keywords

Tbx1; FGF; pharyngeal endoderm; morphogenesis

INTRODUCTION

The T-box transcription factor *Tbx1* is essential for the normal development and morphogenesis of several structures and organs in the head, pharynx and chest (Jerome and

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Papayioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Haploinsufficiency for *TBX1* is associated with DiGeorge/velocardiofacial syndrome, which is most often caused by microdeletions of chromosome 22q11.2 (Scambler, 2010), although isolated mutations in the *TBX1* gene itself have been reported (Yagi et al., 2003; Paylor et al., 2006; Torres-Juan et al., 2007). One of the most striking phenotypic abnormalities in *Tbx1*^{-/-} mouse embryos is a non-segmented caudal pharyngeal apparatus caused by caudal pharyngeal pouch aplasia, which is responsible for defects such as thymus aplasia (Jerome and Papayioannou, 2001; Lindsay et al., 2001; Vitelli et al., 2002a).

The pharyngeal apparatus is a transient structure in the mid-gestation embryo that gives rise to several essential organs (Graham, 2003). This structure is formed by the evagination of a series of pharyngeal pouches from the foregut endoderm and the invagination of the pharyngeal ectoderm to form ectodermal clefts. As the ectodermal clefts contact the endodermal pouches, the pharyngeal region is divided into distinct segments referred to as pharyngeal arches. Each pharyngeal arch contains a pharyngeal arch artery, surrounded by a mesodermal core and neural crest-derived mesenchyme (Graham, 2003). Previous studies have shown that the pharyngeal arches form in an iterative fashion, with the anterior arches forming first, followed by the progressive addition of more caudal arches (Tamarin and Boyde, 1977; Veitch et al., 1999; Crump et al., 2004). In mouse and humans, five distinct pairs of arches can be distinguished (indicated as I, II, III, IV and VI) that are separated by four pairs of pharyngeal pouches (pp1–pp4) (Graham et al., 2005).

Experiments in chick and zebrafish embryos have suggested that cellular processes that direct morphogenesis of the pharyngeal endoderm to form the pharyngeal pouches are the key event that drives the segmentation of the pharyngeal apparatus (Veitch et al., 1999; Crump et al., 2004). The formation of the pharyngeal pouches also provides a permissive niche for neural crest cell migration. In addition to producing inductive signals that guide the migrating crest into the apparatus (Begbie et al., 1999), the evagination toward and fusion of the pharyngeal endoderm with the ectoderm also appears to provide a physical barrier that can influence neural crest infiltration of the pharyngeal arches (Rizzoti and Lovell-Badge, 2007). Despite this apparently critical role for the pharyngeal endoderm, the mechanisms that control pouch morphogenesis are incompletely understood. *Tbx1* appears to be a key player in pharyngeal pouch formation, however, as it is expressed in pharyngeal ectoderm, endoderm and mesoderm, it is not yet known whether *Tbx1* in the endoderm directly controls pouch morphogenesis. In addition to *Tbx1*, experiments in zebrafish have implicated FGFs, in particular *Fgf3* and *Fgf8*, in pouch formation (Crump et al., 2004). Studies in the mouse embryo have shown that pharyngeal pouch formation is disorganized in *Fgf8* hypomorphic embryos (Abu-Issa et al., 2002; Frank et al., 2002). *Fgf3*^{-/-};*Fgf10*^{-/-} mouse embryos exhibit severe hypoplasia of the 4th pharyngeal arch (Urness et al., 2011), and embryos homozygous for a hypomorphic *Fgfr1* mutation have second pharyngeal arch hypoplasia (Trokovic et al., 2003). Together these studies suggest important roles for FGF signaling in pharyngeal segmentation in the mouse. These FGF ligands are produced in both epithelial (endoderm and ectoderm) and mesodermal tissues in the developing pharyngeal region and are likely involved in mediating complex, cross-regulatory interactions between these tissues during pharyngeal morphogenesis. The function of the endodermal *Fgf3* and

Fgf8 expression domains have not been established and the role of FGF receptor activation and FGF signaling in the pharyngeal endoderm is not known.

A number of observations have suggested that *Tbx1* and FGF signaling are functionally linked. Vitelli et al. showed that *Fgf8* expression in the pharyngeal endoderm is lost in *Tbx1*^{-/-} embryos, indicating that *Tbx1* functions upstream of *Fgf8*. Although 4th pharyngeal arch artery hypoplasia is significantly enhanced in *Tbx1*^{+/-};*Fgf8*^{+/-} embryos compared to *Tbx1*^{+/-} and *Fgf8*^{+/-} embryos, pharyngeal segmentation was not assessed in these mutants (Vitelli et al., 2002b). Brown et al. reported that the deletion of *Fgf8* in *Tbx1*-expressing cells with a *Tbx1-Cre* transgenic line resulted in thymus hypoplasia and a range of cardiovascular defects (Brown et al., 2004). A recent study from Vitelli et al. also suggested that reduced *Fgf8* expression contributes to the outflow tract defect in *Tbx1*^{-/-} embryos (Vitelli et al., 2010). The deletion of a conditional *Tbx1* allele from the pharyngeal endoderm using the *Foxg1-Cre* line generated embryos with an un-segmented caudal pharyngeal region that lacked *Fgf3* and *Fgf8* expression (Arnold et al., 2006). However, *Tbx1* is also upstream of FGF genes in the mesoderm and *Tbx1*, *Fgf8* and *Fgf10* genes interact during remodeling of the pharyngeal arch arteries (Aggarwal et al., 2006). Taken together, these studies clearly place *Tbx1* upstream of FGF gene expression and indicate that reduced FGF gene expression or signaling are responsible for some of the cardiovascular and thymus phenotypes associated with *Tbx1* deficiency but the tissue specific requirements have been unclear. In particular, the functional significance of the loss of *Fgf8* gene expression in the pharyngeal endoderm of *Tbx1*-deficient embryos is not known.

To address these questions, we set out to ablate *Tbx1* expression specifically in the pharyngeal endoderm, leaving other expression domains intact. The effect of *Tbx1* deletion on the expression of two FGF ligands, FGF3 and FGF8, and the ability of *Tbx1*-deficient endoderm to respond to FGF signals were investigated.

RESULTS

***Tbx1* expression in the endoderm is required for pharyngeal pouch morphogenesis**

To determine whether *Tbx1* expression in the endoderm is required for pouch morphogenesis, a conditional *Tbx1* allele was recombined in the foregut endoderm using a *Sox17-iCre* line (Engert et al., 2009; Simrick et al., 2011). Efficient and pharyngeal endoderm-specific Cre recombinase activity was demonstrated by X-gal staining of *Sox17-iCre;Tbx1^{fllox/flox};R26R* embryos (Fig. 1A). The absence of Tbx1 protein in the pharyngeal endoderm and maintenance of Tbx1 in pharyngeal mesoderm of *Sox17-iCre;Tbx1^{fllox/flox}* (*Tbx1* conditional knockout or *Tbx1cko*) embryos was confirmed by immunohistochemistry (Fig. 1B,C). *In situ* hybridization for *Pax1* to detect the pharyngeal pouches in whole mount embryos at E10.5, revealed the characteristic *Tbx1*^{-/-} phenotype of a hypoplastic 1st pouch and absent caudal (2nd and 3rd) pouches (Fig. 1D, D', E, E'). As expected for embryos lacking the 3rd pharyngeal pouch, which forms the thymus, all *Tbx1cko* embryos (n=10/10) examined at E17.5 exhibited thymus gland aplasia (Fig. 1F,G). Interrupted aortic arch type B (IAA-B), a phenotype caused by the absence of the left fourth pharyngeal arch artery, was also observed, in agreement with the absence of caudal arches (Fig. 1H,I). Persistent truncus

arterious (PTA) was not observed in any of the *Tbx1cko* embryos analyzed (n=6). These results provide conclusive proof that endodermal *Tbx1* is required for caudal pharyngeal pouch formation and that *Tbx1* expressed in other pharyngeal tissues cannot compensate for the loss of *Tbx1* in the endoderm during this process. However, the absence of a PTA suggests some differences between the *Tbx1*^{-/-} and these mice.

***Tbx1* deletion in the endoderm only partially eliminates *Fgf3* and *Fgf8* expression**

As previous studies suggested that *Fgf3* and *Fgf8* gene expression in the pharyngeal endoderm requires *Tbx1* (Arnold et al., 2006), we visualized FGF gene expression in *Tbx1cko* embryos by *in situ* hybridisation. In agreement with Arnold *et al.*, *Fgf3* and *Fgf8* expression appeared greatly reduced in the pharyngeal endoderm of *Tbx1cko* embryos examined after whole mount *in situ* hybridization (Fig. 2A–D). However, faint *Fgf3* and *Fgf8* expression could be detected, suggesting that expression was reduced or more diffuse, rather than completely absent (Fig. 2A–D, arrows point to expression sites in the caudal pharyngeal region). *In situ* hybridization analyses on sections indicated that *Fgf8* expression was substantially reduced in the endoderm, but not entirely absent (Fig. 2A',A'',B',B''). As expected, *Fgf8* expression in the pharyngeal ectoderm was not changed in these mutants (Fig. 2B', ecto). *Fgf3* expression was maintained in the pharyngeal endoderm of *Tbx1cko* embryos (Fig. 2C',C'',D',D'').

The loss of endodermal *Tbx1* is not sufficient to disrupt normal FGF signaling levels in the pharyngeal apparatus

Although previous studies have shown that the expression of several genes encoding FGF ligands are altered in *Tbx1*-deficient embryos, few reports on the effects of these changes on downstream FGF signaling are available (Vitelli et al., 2010; Simrick et al., 2012). Therefore, to determine whether the reduced expression of *Fgf8* in the endoderm affected FGF signaling in the *Tbx1cko* pharyngeal apparatus, we analysed the expression of two transcriptional read-outs of FGF signaling, *Etv4* and *Etv5* (Klein et al., 2008). Both of these genes are expressed at high levels in the posterior pouch endoderm of the 1st and 2nd pouches and strongly expressed in the evaginating 3rd and 4th pouches (Fig. 3A,A',C,C'). Although distinct caudal pouches could not be distinguished in *Tbx1cko* embryos, presumptive “pouches” could be identified based on gene expression. The expression levels of FGF target genes in these presumptive pouches of *Tbx1cko* embryos were not markedly different from stage-matched, littermate controls (Fig. 3B,B',D,D'). As *in situ* hybridisation does not provide a quantitative measure of gene expression levels, we also quantified the abundance of two additional read-outs of FGF signaling, *Spry1* and *Spry2* by quantitative RT-PCR. The abundance of *Spry1* and *Spry2* transcripts in RNA extracted from micro-dissected pharyngeal regions confirmed that gene expression was not significantly altered in *Tbx1cko* embryos (Fig. 3E). This observation is in contrast to the severely reduced *Spry1* and *Spry2* expression in the pharyngeal region of *Tbx1*^{-/-} embryos (Simrick et al., 2012). Taken together, these observations indicate that the near-complete loss of *Fgf8* expression in the endoderm of *Tbx1cko* embryos is not sufficient to cause an over-all reduction in FGF signaling levels in the developing pharyngeal region at the time of caudal pouch formation. Importantly, these data suggest that the failure of pharyngeal segmentation in *Tbx1cko*

embryos cannot be attributed to a reduction in FGF signaling levels in the pharyngeal endoderm.

***Fgf8* deletion in the pharyngeal endoderm is not sufficient to prevent pharyngeal pouch formation**

A prediction from the results presented thus far is that the deletion of *Fgf8* from the pharyngeal endoderm would not be sufficient to cause pharyngeal segmentation defects. To test this, we generated *Sox17-iCre;Fgf8^{lox/flox}* (*Fgf8cko*) embryos. *Pax1* *in situ* hybridisation at E10.5 revealed normal pharyngeal segmentation in *Fgf8cko* embryos (Fig. 3F,G). Although pouch formation was not completely abolished in *Fgf8cko* mutants as in *Tbx1cko* embryos, the caudal pouches appeared slightly hypoplastic in some embryos, although we considered these to be within normal inter-embryonic variation (compare the size of pp3 in Fig. 3F with G; Table 1). To determine whether *Fgf8* deletion resulted in thymus hypoplasia, *Fgf8cko* embryos were examined at E17.5. No thymus hypoplasia was observed, consistent with normal third pharyngeal pouch development (Fig. 3H).

Endodermal *Fgf3* and *Fgf8* function redundantly during pharyngeal segmentation

The observations that *Fgf3* expression is maintained in the pharyngeal endoderm of *Tbx1cko* embryos (Fig. 2D') and that pharyngeal segmentation proceeds normally in *Fgf8cko* embryos (Fig. 3G), raise the possibility that the *Fgf3* expression that remains in the *Tbx1cko* endoderm compensates for the loss of *Fgf8*. To address this possibility, we analysed embryos in which different combinations of *Fgf3* or *Fgf8* conditional alleles were deleted in the endoderm with the *Sox17-iCre* line (Suppl. Fig. 1). Significant abnormalities in the pharyngeal apparatus were present when at least three FGF alleles were deleted from the endoderm (Compare Fig. 4C–E with Fig. 4A,B). The most consistent anomaly was hypoplasia of the 2nd pharyngeal arch (Table 1). In some cases the second arch hypoplasia was so severe that the 1st and 2nd pharyngeal pouches, visualized by *Pax1* expression, appeared fused together (see Fig. 4D).

In contrast to *Tbx1cko* embryos, caudal pharyngeal pouches were still present when both *Fgf3* and *Fgf8* were deleted, although the pouches were clearly hypoplastic in these embryos (Fig. 4E, Table 1). These data indicate that *Fgf3* expression in the endoderm to some extent compensates for the loss of *Fgf8* expression. However, the combined deletion of *Fgf3* and *Fgf8* from the endoderm does not phenocopy *Tbx1cko* embryos. Rather, the phenotypes observed in *Fgf3;Fgf8cko* embryos are reminiscent of other examples in which FGF signaling and related pathways have been disrupted in the pharyngeal region (Trokovic et al., 2003; Trokovic et al., 2005; Rizzoti and Lovell-Badge, 2007; Kameda et al., 2009). In particular, 2nd arch hypoplasia has been attributed to defects in FGF-dependent patterning of the pharyngeal epithelia.

FGF signaling in the endoderm is required for normal development of the pharyngeal apparatus

If our hypothesis that the caudal pouch aplasia in *Tbx1cko* mutants are not caused by the reduced FGF signaling within the pharyngeal endoderm, rendering the endoderm unresponsive to FGF signals, should result in a phenotype similar to FGF mutants and not

pouch aplasia as observed in *Tbx1cko* embryos. We therefore investigated whether the simultaneous deletion of the two main FGF receptors expressed in the pharyngeal region, *Fgfr1* and *Fgfr2* (Trokovic et al., 2005), with *Sox17-iCre* phenocopied *Tbx1cko* or *Fgf3;Fgf8cko* mutants. The analysis of pouch formation in these embryos indicated that the near-loss of FGF signaling in the pharyngeal endoderm resulted in 2nd arch and caudal (3rd pouch) hypoplasia (Fig. 4F,G, Table 1). To assess the impact of deleting *Fgfr1* and *Fgfr2* in the endoderm on FGF signaling in this tissue, we visualized *Etv4* and *Etv5* expression in these *Fgfr1/2cko* embryos. *Etv4* and *Etv5* expression were markedly reduced in the endoderm (Fig. 4H–K, indicating that 1) these genes are *bona fide* readouts of FGF signaling in the pharyngeal endoderm and 2) that deleting *Fgfr1* and *Fgfr2* was sufficient to dramatically reduce the responsiveness of this tissue to FGF ligands.

In conclusion, our analysis of mutant lines in which either multiple FGF ligands or FGF receptors were deleted in the endoderm confirm that FGF signaling is required for normal pharyngeal development and indicates that the pharyngeal endoderm itself needs to be responsive to FGF signaling for this process. Taken together with other published reports, our observations indicate that the abnormalities in pharyngeal development in embryos in which FGF signaling has been disrupted, are strikingly similar to each other, and involves hypoplasia of the 2nd pharyngeal arch, irregular pouch formation and pouch hypoplasia (Trokovic et al., 2003; Trokovic et al., 2005; Hoch and Soriano, 2006; Kameda et al., 2009). Most critically, the pharyngeal abnormalities in FGF pathway mutants are phenotypically distinct from the characteristic loss of caudal pouch formation observed in *Tbx1*^{-/-} and endodermal *Tbx1cko* embryos (Fig. 1D). This conclusion is in agreement with the findings reported above which suggest that the defects in caudal pouch formation and pharyngeal segmentation in *Tbx1*-deficient embryos are not associated with significant perturbations in FGF signaling.

The pharyngeal endoderm maintains its capacity to proliferate in the absence of *Tbx1*

The analysis of cellular defects in embryos in which *Tbx1* was conditionally ablated at different developmental time points, has revealed a significant deficit in the proliferation of the pharyngeal endoderm by E10 (Xu et al., 2005). To determine whether this effect on cell proliferation is directly caused by loss of *Tbx1* function in the endoderm, we visualized mitotic cells with an antibody to Ser10-phospho-histone H3 and calculated the mitotic index in the pharyngeal region of *Tbx1cko* embryos in the same way as Xu *et al.* (Xu et al., 2005). We failed to detect any significant difference in cell proliferation in the mutant endoderm (Fig. 5). This finding suggests that a drastic change in cell proliferation at E9.5 could not explain the failure of pharyngeal pouch evagination observed in *Tbx1cko* embryos at this stage of development.

DISCUSSION

Reviewing the link between *Tbx1* and FGF signaling in the endoderm

Previous studies have provided compelling evidence that *Tbx1* functions upstream of a number of FGF genes (Vitelli et al., 2002b; Brown et al., 2004; Aggarwal et al., 2006; Arnold et al., 2006; Vitelli et al., 2010). In particular, the observation that *Fgf8* expression is

lost in the pharyngeal endoderm of *Tbx1*^{-/-} embryos, taken together with the demonstration that *Tbx1* and *Fgf8* null alleles interact during pharyngeal development, provided strong evidence for a functional link between Tbx1 and the FGF signaling pathway (Vitelli et al., 2002b). However, to what extent the reduced FGF signaling in *Tbx1*-deficient embryos is responsible for the phenotypes associated with *Tbx1* deficiency has not been examined fully.

In the present study we describe the generation of endoderm-specific *Tbx1* conditional mutants, and confirm *Fgf8* downregulation in *Tbx1*-deficient endoderm. Despite this *Fgf8* downregulation, we found that FGF signaling was maintained at normal levels in the endoderm. Furthermore, we could show that *Fgf8* deletion from the endoderm was not sufficient to cause caudal pouch agenesis. These observations indicate that the loss of *Fgf8* expression in the endoderm of *Tbx1*-deficient embryos is by itself not responsible for the caudal pharyngeal pouch agenesis present in these embryos. The deletion of both *Fgf3* and *Fgf8*, or *Fgfr1* and *Fgfr2*, were sufficient to downregulate FGF signaling in the endoderm and cause pharyngeal phenotypes. However, these “FGF” phenotypes were qualitatively distinct from the *Tbx1* phenotype, such that no caudal pouch aplasia was observed. Taken together, these findings suggest that the mechanisms whereby *Tbx1* in the endoderm controls caudal pouch formation are distinct from its effects on FGF signaling. Our recent demonstration that *Tbx1* haplo-insufficiency can also affect the ability of the embryo to resist developmental alterations caused by increased FGF signaling, further suggest that the relation between *Tbx1* and the FGF pathway may be more complex than originally anticipated (Simrick et al., 2012).

Tissue-specific requirements for *Tbx1* during pharyngeal segmentation

The tissue-specific roles of *Tbx1* have been dissected using a number of Cre lines with different tissue specificities. Together with a previous study, the data presented here identifies a critical role for endodermal *Tbx1* in caudal pharyngeal pouch formation (Arnold et al., 2006). The pharyngeal ectoderm appears to not be required for pouch morphogenesis and pharyngeal segmentation (Zhang et al., 2005; Calmont et al., 2009). Intriguingly, Zhang et al. showed that the deletion of *Tbx1* in the mesoderm was sufficient to cause caudal pharyngeal segmentation defects and thymus aplasia. Furthermore, restoring *Tbx1* expression in the mesoderm of *Tbx1* hypomorphic embryos partially rescued the caudal pouch phenotypes although it failed to rescue thymus aplasia (Zhang et al., 2006b). Thus, it appears that mesodermal *Tbx1* has a role in pouch formation. However, the exact relation between the mesoderm and endoderm during this process remain incompletely understood.

FGF signaling in the endoderm is critical for 2nd arch expansion

Our analysis of various compound, endoderm-specific conditional FGF mutants confirms crucial roles for FGF signaling in pharyngeal development. The most consistent phenotypic feature of these mutants is second arch hypoplasia, in agreement with a previous study (Trokovic et al., 2003). Our observation that the deletion of both *Fgf3* and *Fgf8* from the pharyngeal endoderm resulted in similar phenotypes to the deletion of FGF receptors from the endoderm requires further investigation. As FGF3 has high affinity for FGF receptor isoforms expressed in epithelia (FGFR1(IIIb) and FGFR2(IIIb)), it is conceivable that FGF3 may regulate pharyngeal pouch formation in an autocrine fashion. However, FGF8

preferentially signals to FGFR2(IIIc) and FGFR4, suggesting that it may regulate endodermal behavior indirectly through a relay via the mesenchyme (Zhang et al., 2006a). Our current experimental system cannot distinguish between these possibilities, especially given the high level of functional redundancy between the different FGF ligands present in the developing pharyngeal region.

In summary, our study confirms that endodermal *Tbx1* expression is necessary for caudal pouch formation. Whilst FGF signaling in the pharyngeal endoderm is also required for normal pouch morphogenesis, the deletion of *Tbx1* from the endoderm does not affect FGF signaling levels. Thus, we conclude that *Tbx1* directs pouch formation in an FGF-independent manner.

EXPERIMENTAL PROCEDURES

Mouse husbandry, embryo collection and genotyping

The *Sox17-2A-iCre (Sox17-iCre)* transgenic line was used to delete a conditional *Tbx1^{fllox}* allele in the pharyngeal endoderm (Arnold et al., 2006; Engert et al., 2009). *Sox17-iCre;Tbx1^{fllox/+}* males were mated with *Tbx1^{fllox/flox}* females. *Sox17-iCre;Tbx1^{fllox/flox}* embryos lacked *Tbx1* in the endoderm and are referred to as *Tbx1* conditional knockout (*Tbx1cko*) embryos. Cre-negative *Tbx1^{fllox/+}* and *Tbx1^{fllox/flox}* littermates were used as controls in all experiments. In addition to *Tbx1*, *Fgf8* (Meyers et al., 1998; Moon and Capecchi, 2000), *Fgf3* (Urness et al., 2011), *Fgfr1* (Xu et al., 2002) and *Fgfr2* (Yu et al., 2003) conditional alleles were deleted in the pharyngeal endoderm by a *Sox17-iCre* line using the same breeding strategy, with the exception that males were heterozygous for two conditional alleles e.g. *Sox17-iCre;Fgfr1^{fllox/+};Fgfr2^{fllox/+}*; and females homozygous for two conditional alleles e.g. *Fgfr1^{fllox/flox};Fgfr2^{fllox/flox}*. During the analysis of *Fgf3;Fgf8* conditional mutants, *Sox17-iCre;Fgf3^{-/+};Fgf8^{-/+}* males (carrying an *Fgf3* and *Fgf8* null () allele), were mated with *Fgf3^{fllox/flox};Fgf8^{fllox/flox}* or *Fgf3^{-/fllox};Fgf8^{fllox/flox}* females. Mouse lines were maintained on a mixed (C57BL/6J; FVB/N) genetic background. *Sox17-iCre;Tbx1^{fllox/+}*, *Sox17-iCre;Fgf8^{fllox/+}* and *Sox17-iCre;Fgfr1^{fllox/+};Fgfr2^{fllox/+}* embryos displayed no overt pharyngeal pouch or endocrine abnormalities indicating that *Sox17-iCre* alone did not cause pharyngeal phenotypes. Noon of the day when a vaginal plug was detected was considered E0.5. Embryos were staged more precisely by counting somites and only stage-matched embryos were compared in experiments. Embryos were fixed and processed as appropriate (see below), or pharyngeal tissues were microdissected for RNA extraction. Genotyping of the various alleles were performed by PCR using yolk sac DNA as template and primer pairs described in the original publications.

In situ hybridization (ISH)

Embryos were fixed overnight at 4°C in 4% paraformaldehyde in RNase-free PBS. For ISH on sections, embryos were dehydrated through a series of ethanol and histoclear washes and embedded in wax. Alternate 7µm sections were cut and dried overnight at 42°C. ISH was performed according to standard methods (Yaguchi et al., 2009). For ISH on whole embryos, E10.5 embryos were dissected in RNase free PBS, fixed overnight in 4% PFA at 4°C, processed and hybridised with digoxigenin-labelled antisense RNA probes as

previously described (Wilkinson et al., 1989). The probes used for ISH have all been previously described as follows: *Fgf3* (Robinson et al., 1998), *Fgf8* (Crossley and Martin, 1995), *Etv4*, *Etv5* (Klein et al., 2006) and *Pax1* (Deutsch et al., 1988).

Morphological analysis of thymi

The thoracic cavity of E17.5 embryos were opened and photographed under a stereomicroscope to score the presence/absence of thymus lobes above the heart. Image J was used to measure the circumference of each thymus lobe as an estimate of thymus size. Statistical analysis (Student's t-test) was done using Graph Pad software.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on 7 μ m, wax-embedded sections that were cleared of wax in Xylene and rehydrated through a series of ethanol:PBS (100%, 95%, 90%, 70%) washes. After a final wash in PBS, antigen retrieval was performed by microwaving the slides in a 10mM sodium citrate solution, followed by a wash in PBT₂ (0.2% Tween-20 in PBS). A blocking solution of 10% goat serum in PBT₂ was applied for a minimum of 1 hour before application of the primary antibody (diluted in a solution of 5% goat serum in PBT₂) and incubation at 4°C overnight in a humidified chamber. Primary antibodies and dilutions were: rabbit anti-phospho-Histone H3(Ser10) (PH3) (Cell Signaling 9701, 1:250 dilution) to identify cells in mitosis, and mouse anti-E-cadherin (Fitzgerald #02660, 1:200) to label epithelial tight junctions. Subsequently, the primary antibody was removed and the slides washed in PBT₂ three times before application of an Alexafluor-conjugated secondary antibody, (Invitrogen, diluted 1:250 in a solution of 5% goat serum in PBT₂), in a humidified chamber for a minimum of 1 hour. The secondary antibody solution was removed and the slides washed in PBT₂. After a final wash in PBS with Hoechst 33342 (Invitrogen, H3570/23363w, 1:50,000), slides were mounted in AF100 mounting solution (Citifluor).

Proliferation assay

To determine the level of proliferation in the pharyngeal epithelia, tissue sections were labeled with antibodies raised against PH3 and E-cad. PH3⁺ cells were counted in the pharyngeal endoderm and ectoderm (more than 2000 E-cad positive cells per tissue), and a mitotic index (MI) calculated as described (Xu et al., 2005):

$$MI = \frac{\text{Number of proliferating cells (PH3 positive)}}{\text{Total cell number}}$$

Bar graphs and p-values (calculated using the Student's t-test) were produced with Graph Pad software.

Quantitative RT-PCR

The pharyngeal apparatus (from pharyngeal arch I–VI) was microdissected, heart and neural tube tissues removed, and total RNA extracted and genomic DNA removed using the Absolutely RNA Microprep Kit (Agilent Technologies). A total of 200ng of RNA was used

for first-strand DNA synthesis with nanoScript Precision RT kit (PrimerDesign Ltd.) according to the manufacturer's specifications. cDNA synthesis reactions without reverse transcriptase enzyme (no RT) were used as controls for q-RT-PCR. qRT-PCR was performed on a RotorGene Q cycler (Qiagen) using Precision qPCR MasterMix kit (PrimerDesign Ltd.). Appropriate normalising genes were detected using qbasePLUS software (Biogazelle) with geNorm reference kits (PrimerDesign Ltd.) All reactions were performed in triplicate and normalised to GAPDH. Cq threshold values were determined manually and all were at least 5 Cq values below no RT controls. Cq values were calculated relative to stage-matched control samples using Microsoft Excel software and graphs were produced using GraphPad Prism software. Primers were designed using primer blast (NCBI) and are as follows: *Gapdh* as a normaliser (AGGTCGGTGTGAACGGATTG and TG TAGACCATGTAGTTGAGGTCA), *Spry1* (GCTCGTGGCTGTCCATCT and GAAACACGTGAGTCCCTTGC) and *Spry2* (TGGTGCAAAGCCGCGATCAC and GCAAAGGCTGCGACCCGTTG).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Embryos that lack TBX1 in the pharyngeal endoderm fail to form caudal pharyngeal pouches.
- *Fgf8* expression is greatly reduced in TBX1-deficient endoderm.
- The deletion of *Fgf8* from the endoderm does not perturb pouch development.
- TBX1 and FGF signaling deficiency in the endoderm result in distinct phenotypes.

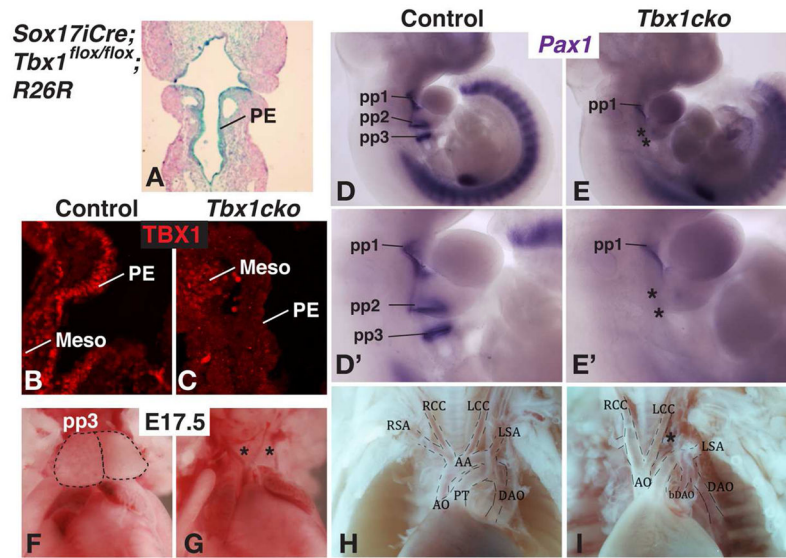


Figure 1. *Tbx1* deletion from the pharyngeal endoderm results in caudal pharyngeal pouch agenesis and thymus aplasia

A) Frontal section through a *Sox17-iCre;Tbx1^{flox/flox};R26R* E9.5 embryo stained with X-gal (blue) and counterstained with Fast Red. Note the strong Cre-activated β -galactosidase activity in the pharyngeal endoderm (PE).

B,C) TBX1 immunohistochemistry on frontal sections through the pharyngeal region of E9.5 mouse embryos. Nuclear TBX1 is present in the pharyngeal endoderm (PE) of the control embryo (A), and absent from the PE of the *Tbx1cko* embryo (B). TBX1 protein is still present in the mesodermal core (Meso) of the *Tbx1cko* embryo.

D,E) Wholemount E10.5 embryos after in situ hybridization with an antisense RNA probe to detect *Pax1* mRNA, which is expressed in the pharyngeal pouches. Pharyngeal pouches 1–3 (pp1–pp3) are labeled in the control embryo (D). Only pp1 is present in the *Tbx1cko* embryo, with asterisks indicating agenesis of pp2–pp3 (E).

D',E') Magnified views of the pharyngeal apparatus of embryos in D and E.

F,G) Thoracic cavities of E17.5 embryos with the thymus lobes outlined in broken lines in the control (F) and thymus aplasia indicated by asterisks in the mutant (G).

H,I) The heart and its associated vessels imaged *in situ* within the chest cavity of E17.5 embryos. Interrupted aortic arch type B (IAA-B) is indicated by an asterisk (*).

PTA (scored as a single vessel arising from the heart) was not evident in any *Tbx1cKO* embryos analyzed, n=6.

PT = Pulmonary trunk, R/LCC = Right/Left common carotid, R/LSA = Right/Left Subclavian, A0 = Aorta, AA = Aortic arch, DAO = Descending aorta, bDAO = branch of the descending aorta.

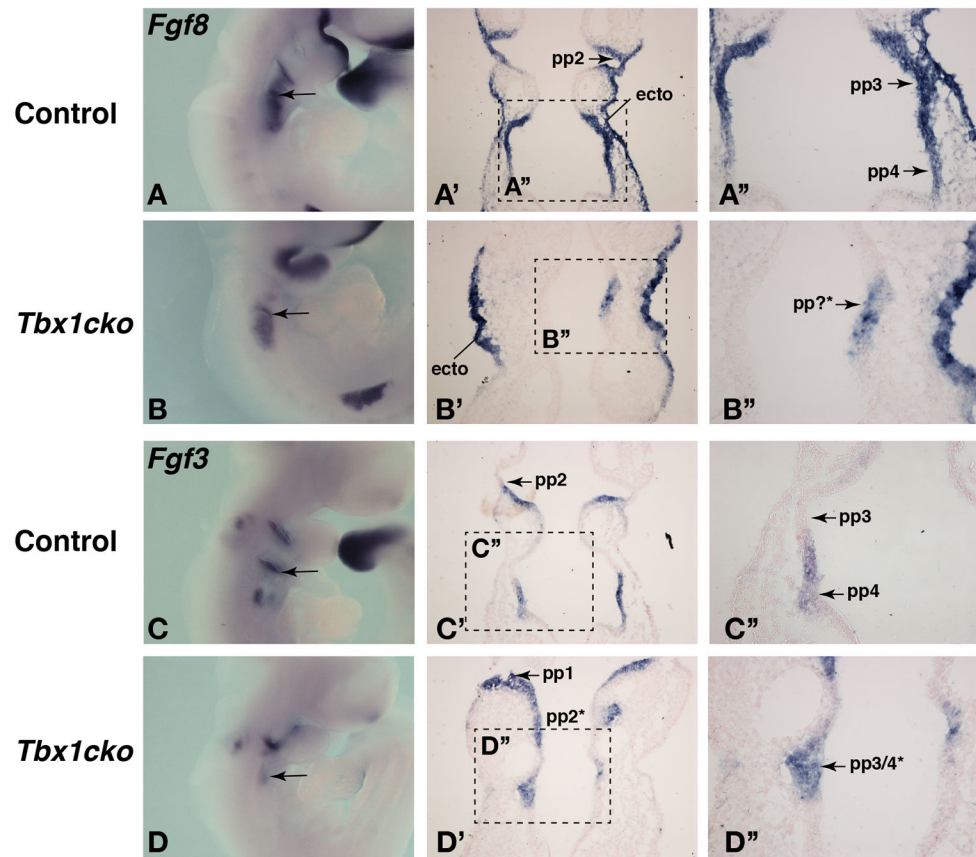


Figure 2. *Fgf8*, but not *Fgf3* gene expression is reduced in the *Tbx1*-deficient endoderm
 A,B) Wholemount *Fgf8* in situ hybridization on E9.5 embryos, anterior to the right. Arrows point to expression domains in the caudal pharyngeal region.
 A',B') *Fgf8* gene expression visualized by in situ hybridization on frontal sections through the pharyngeal region of E9.5 embryos. *Fgf8* expression in pp2 of the control (A') and pharyngeal ectoderm (ecto) of both embryos is indicated.
 A'',B'') Magnified views of the areas indicated in panels A and B. *Fgf8* expression in the evaginating pp3 and pp4 are indicated in the control (A'') and a small patch of cells with low *Fgf8* expression is visible in a presumptive pouch (pp?*) of the mutant endoderm (B'').
 C,D) Wholemount *Fgf3* in situ hybridization on E9.5 embryos, anterior to the right. Arrows point to expression domains in the caudal pharyngeal region.
 C',D') *Fgf3* gene expression visualized by in situ hybridization on frontal sections through the pharyngeal region of E9.5 embryos. *Fgf3* expression in pp2 of the control (C') and pp1 and the presumptive pp2 (pp2*) of the mutant embryo (D') are indicated.
 C'',D'') Magnified views of the areas indicated in panels C and D. *Fgf3* expression in the evaginating pp4 is indicated in the control (C'') and equivalent levels of *Fgf3* expression in a presumptive pouch (pp3/4*) of the mutant endoderm can be seen (D'').

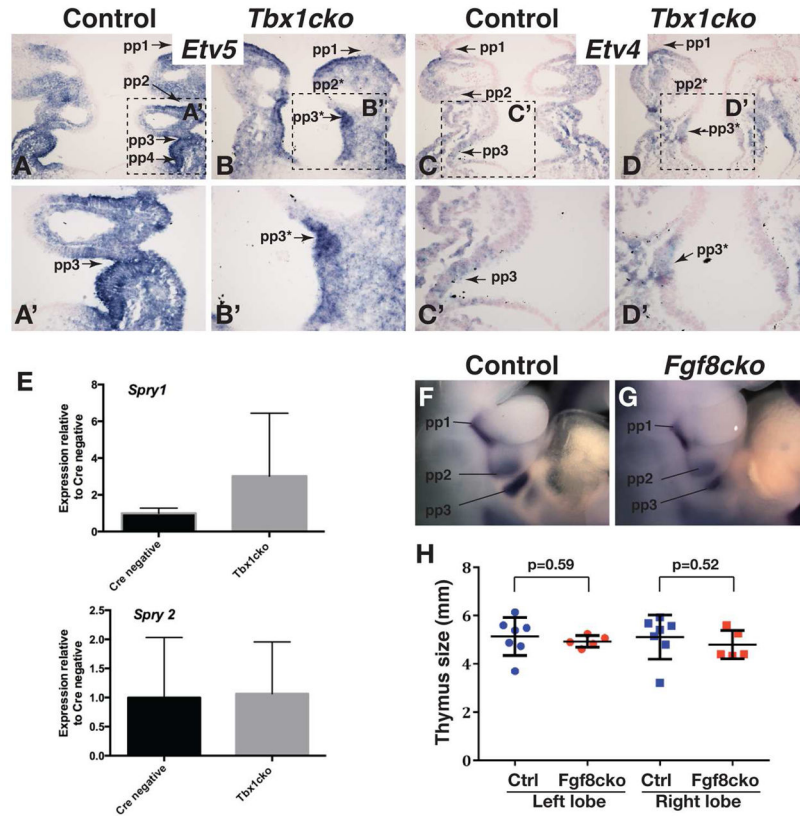


Figure 3. Normal levels of FGF signaling is maintained in *Tbx1cko* embryos

A–D) The expression of two FGF signaling reporter genes, *Etv5* (A,B) and *Etv4* (C,D) in frontal sections through E9.5 pharyngeal apparatus are compared between control (A,C) and mutant (B,D) embryos. Pharyngeal pouches (pp1–pp4) and presumptive pouches (pp2*, pp3*) are indicated.

A'–D') Magnified views of the areas indicated in panels A–D. Note the equivalent levels of gene expression in evaginating and presumptive 3rd pouches of control and mutant embryos. E) Relative qRT-PCR quantification of FGF-regulated transcripts in the E9.5 pharyngeal region.

F,G) *Pax1* in situ hybridization to visualize pharyngeal pouches in control (F) and *Fgf8cko* (G) embryos at E10.5. Note that of all pharyngeal pouches are present in the mutant.

H) Estimated sizes of thymus lobes at E17.5 as measured by thymus circumference from wholemount pictures. Note the absence of any significant thymus hypoplasia in *Fgf8cko* embryos.

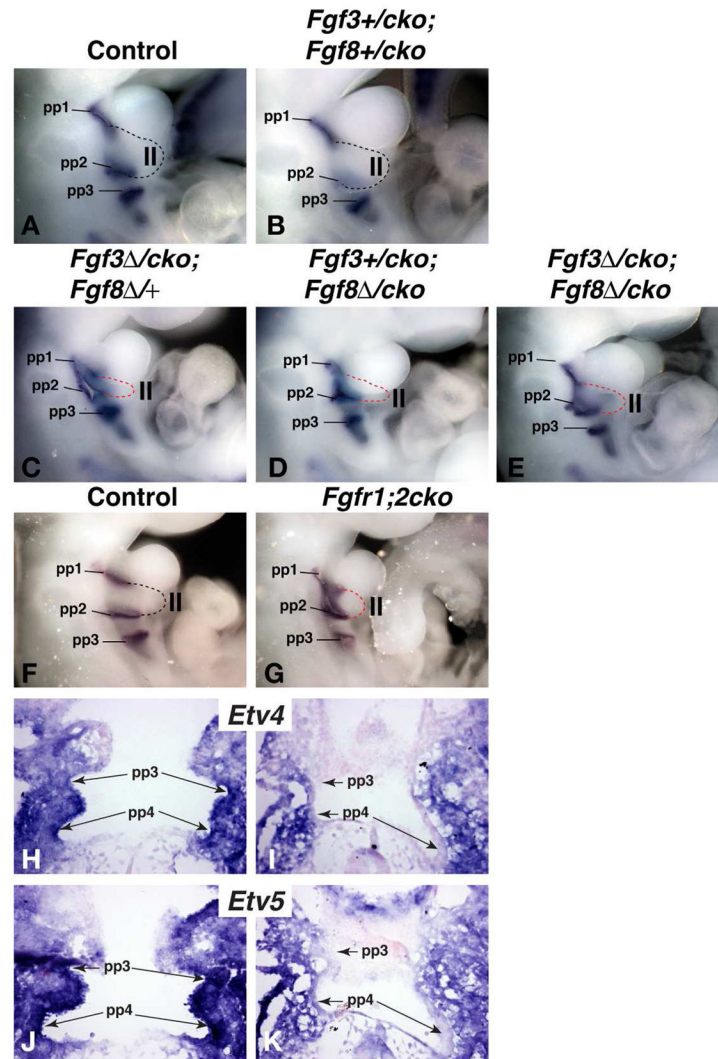


Figure 4. Endodermal deletion of *Fgf3* and *Fgf8*, or *Fgfr1* and *Fgfr2* disrupt normal pharyngeal morphogenesis

A–E) Visualization of pharyngeal pouches in E10.5 embryos by *Pax1* in situ hybridization. *Fgf3*^{+/cko};*Fgf8*^{+/cko} embryos (B) have normal-sized second pharyngeal arches (II, outlined in broken lines), compared to control embryos (A). Embryos with only one remaining *Fgf3* or *Fgf8* allele (C,D) or complete loss of all *Fgf3* and *Fgf8* alleles in the endoderm (E), exhibit 2nd arch and 3rd pharyngeal pouch (pp3) hypoplasia (also see Table 1).

F,G) Wholemount *Pax1* in situ hybridization of E10 embryos reveals pharyngeal phenotypes identical to *Fgf3*;*8cko* embryos in embryos that lack both *Fgfr1* and *Fgfr2* in the endoderm (G).

H–K) Comparison of *Etv4* and *Etv5* expression in control (H,J) and *Fgfr1*;*2cko* (I,K) embryos at E9.5, reveal a significant reduction of FGF signaling levels in the FGFR-deficient pharyngeal endoderm.

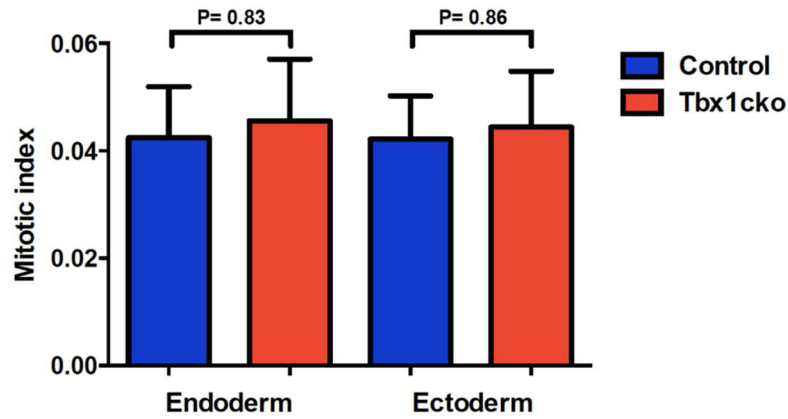


Figure 5. *Tbx1* deletion from the endoderm does not affect cell proliferation

Mitotic indices (number of proliferating (phosphor-histone H3-positive) cells/total cell number) in pharyngeal endoderm and ectoderm of control and *Tbx1cko* embryos at E9.5. More than 2000 cells were counted for each cell type in each embryo. The data is representative of two independent experiments.

Table 1
Summary of pharyngeal apparatus defects in conditional FGF-deficient embryos

Genotype	2nd arch hypoplasia			3rd pouch hypoplasia		
	Unilateral	Bilateral	Total incidence/pouch	Unilateral	Bilateral	Total incidence/pouch
Cre-negative	0/12	0/12	0/24	0/12	0/12	0/24
<i>Fgf8^{cko}</i>	0/3	0/3	0/6	0/3	0/3	0/6
<i>Fgf3^{+/cko}; Fgf8^{+/cko}</i>	0/2	0/2	0/4	0/2	0/2	0/4
<i>Fgf3^{/cko}; Fgf8^{/+}</i>	1/4 (L)	0/4	1/8	0/4	0/4	0/8
<i>Fgf3^{/+}; Fgf8^{/cko}</i>	2/2 (R)	0/2	2/4*	1/2 (L)	0/2	1/4
<i>Fgf3^{/cko}; Fgf8^{/cko}</i>	1/4 (L)	2/4	5/8***	1/4 (R)	1/4	3/4*
<i>Fgfr1;2cko</i>	0/2	2/2	4/4***	0/2	2/2	4/4***

Phenotypes determined from whole mount images of E10–10.5 embryos after *in situ* hybridization with a *Pax1* antisense probe.

(L) Left side affected.

(R) Right side affected.

* p<0.05,

** p<0.001 (compared to Cre-negative control); Fisher's exact test.