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Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1

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Abstract

Previous studies have suggested a role of the homeodomain Six family proteins in patterning the developing vertebrate head that involves appropriate segmentation of three tissue layers, the endoderm, the paraxial mesoderm and the neural crest cells; however, the developmental programs and mechanisms by which the Six genes act in the pharyngeal endoderm remain largely unknown. Here, we examined their roles in pharyngeal pouch development. *Six1*$^{-/-}$ mice lack thymus and parathyroid and analysis of *Six1*$^{-/-}$ third pouch endoderm demonstrated that the patterning of the third pouch into thymus/parathyroid primordia is initiated. However, the endodermal cells of the thymus/parathyroid rudiments fail to maintain the expression of the parathyroid-specific gene *Gcm2* and the thymus-specific gene *Foxn1* and subsequently undergo abnormal apoptosis, leading to a complete disappearance of organ primordia by E12.5. This thus defines the thymus/parathyroid defects present in the *Six1* mutant. Analyses of the thymus/parathyroid development in *Six1*$^{-/-}$;*Six4*$^{-/-}$ double mutant show that both *Six1* and *Six4* act synergistically to control morphogenetic movements of early thymus/parathyroid tissues, and the threshold of *Six1*/*Six4* appears to be crucial for the regulation of the organ primordia-specific gene expression. Previous studies in flies and mice suggested that Eya and Six genes may function downstream of Pax genes. Our data clearly show that *Eya1* and *Six1* expression in the pouches does not require *Pax1*/*Pax9* function, suggesting that they may function independently from *Pax1*/*Pax9*. In contrast, *Pax1* expression in all pharyngeal pouches requires both *Eya1* and *Six1* function. Moreover, we show that the expression of *Tbx1, Fgf8* and *Wnt5b* in the pouch endoderm was normal in *Six1*$^{-/-}$ embryos and slightly reduced in *Six1*$^{-/-}$; *Six4*$^{-/-}$ double mutant, but was largely reduced in *Eya1*$^{-/-}$ embryos. These results indicate that Eya1 appears to be upstream of very early events in the initiation of thymus/parathyroid organogenesis, while Six genes appear to act in an early differentiation step during thymus/parathyroid morphogenesis. Together, these analyses establish an essential role for *Eya1* and Six genes in patterning the third pouch into organ-specific primordia.

Keywords

Six1; *Six4*; Third pharyngeal pouch; Patterning; Thymus; Parathyroid; Eya1; Pax; *Hoxa3*; Fox; *Tbx1*; Fgf8; Wnt5b

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Introduction

Pharyngeal endoderm is an important tissue for patterning the vertebrate head. The endoderm of the pharynx evaginates or outgrows toward the ectoderm to form segmental pouches, which develop in an anterior to posterior sequence and are separated from each other by pharyngeal arches. The establishment of the anterior to posterior endodermal segmentation appears to be independent of cephalic neural crest cells (Veitch et al., 1999; Piotrowski and Nusslein-Volhard, 2000; Trokovic et al., 2003). However, it is unclear whether the endoderm segments autonomously or whether its segmentation is induced by surrounding tissues.

The pharyngeal pouches are transient embryonic structures and give rise to craniofacial organs, including the thymus and parathyroid glands. During the segmentation of the pharynx, endodermal cells of the prospective third pharyngeal pouch receive the signals that provide the initial positional information for thymus and parathyroid development. Several studies including chick-quail chimera experiments and cell lineage analysis in mice have provided clear evidence that the endoderm provides the initiating signals for induction of the thymus and is sufficient for the development of the major epithelial subtypes (LeDouarin and Joterean, 1975; Manley and Blackburn, 2003). However, its subsequent development depends on proper interaction of the endoderm with surrounding mesenchyme. The parathyroid is also derived from the third pharyngeal pouch and it develops concurrently with the thymus. The prospective thymus and parathyroid regions in the common primordia of third pouch begin to separate from E12.5 when the thymic epithelial rudiment is encapsulated by mesenchyme. At present, the mechanisms controlling the initial formation of the common primordium and its subsequent development into thymus and parathyroid are unclear.

The prospective thymus and parathyroid are marked by the expression of the transcription factors Foxn1 and Gcm2, respectively (Gordon et al., 2001). Inactivation of the forkhead transcription factor Foxn1 leads to a very severe form of thymic hypoplasia and the complete absence of thymopoiesis (Nehls et al., 1994; Su et al., 2003), while loss of *Gcm2*, a mouse homolog of *Drosophila Glial cells missing* gene, results in aparathyroid (Gunther et al., 2000). Gene inactivation and in situ hybridization experiments revealed several other transcription factors in a role in mediating the formation of the thymus and parathyroid. *Hoxa3* is expressed in both the third pouch endoderm and the neural crest mesenchyme and has been suggested to function upstream of *Pax1* and *Pax9* (Su et al., 2001). Mutations in each of these three genes in mice result in aparathyroid and athymia or hypoplastic parathyroid and thymus (Manley and Capecchi, 1995; Wallin et al., 1996; Peters et al., 1998). The T-box transcription factor Tbx1 is normally expressed in the pharyngeal endoderm and the arch mesenchyme as well as in the developing thymus at later stages (Garg et al., 2001). Its expression in the pharyngeal region appears to be regulated by Sonic hedgehog (Shh) signaling and mutations in this gene cause thymus and parathyroid defects (Garg et al., 2001; Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001; Abu-Issa et al., 2002). The *eyes absent* gene *Eya1*, which encodes a transcription coactivator, is expressed early in the pharyngeal endoderm, mesenchyme and ectoderm and *Eya1* knockout mice had no thymus and parathyroid (Xu et al., 2002). In addition to these transcription factors, several signaling pathways are essential for normal development of the thymus. Several studies have suggested that early endodermal segmentation depends on retinoic acid signaling already in the cephalochordates (Wendling et al., 2000; Escriva et al., 2002). Recently, Wnt signaling has been shown to regulate *Foxn1* expression (Balciunaite et al., 2002). Several other signaling pathways including Fibroblast growth factors (Fgfs), Transforming growth factors (Tgfs) and Shh are also implicated in thymic development (Abu-Issa et al., 2002; Crump et al., 2004; Moore-Scott and Manley, 2004). Although these

studies have started to define specific genes controlling early thymus/parathyroid development, the identity of the regulatory pathways and the molecular basis of epithelial–mesenchymal interactions are largely unknown.

We have previously shown that *Six1*, a member of the homeobox Six gene family homologous to *Drosophila sine oculis (so)* gene, is expressed in the pharyngeal endoderm, mesenchyme and ectoderm from early stages and its expression in the endoderm and ectoderm is *Eya1*-dependent (Xu et al., 2002). *Six1* knockout mice also lack thymus (Laclef et al., 2003a). Mutations in the human *EYA1* or *Six1* gene cause Branchio-Oto-Renal (BOR) syndrome and Branchio-Oto (BO) syndromes, autosomal dominant disorders with incomplete penetrance and variable combinations of branchial, otic and renal anomalies (Abdelhak et al., 1997a,b; Vincent et al., 1997; Kumar et al., 1998; Ruf et al., 2004). In addition to hearing loss, present in ~93% of BOR or BO patients, these individuals exhibit branchial arch anomalies including cervical fistulas, sinuses and cysts (Fraser et al., 1980; Chen et al., 1995; Smith and Schwartz, 1998). The Six gene family consists of 6 members (*Six1–6*) and they are suggested to interact with Pax and Eya genes based on their wide coexpression in many tissues during mammalian organogenesis and development (Oliver et al., 1995a,b; Kawakami et al., 1996; Chen et al., 1997; Pignoni et al., 1997; Xu et al., 1997a,b, 1999a,b, 2002a,b, 2003; Zheng et al., 2003; Zou et al., 2004). However, their functional roles during mammalian thymus and parathyroid development have not been studied.

In this study, we examined the role of *Six1* in early pharyngeal organ development. Our analyses show that *Six1* is expressed in third pouch derivatives but not in fourth pouch derivatives, differing from *Eya1* expression in both structures. Our data indicate that the thymus/parathyroid organogenesis defects in the *Six1* mutant appear to represent a block in an early differentiation step. In addition, our data show that the threshold of *Six1*/*Six4* is crucial for the regulation of the organ primordia-specific gene expression, suggesting a functional redundancy between these two genes. Our molecular and phenotypic analyses indicate that Eya1 appears to be upstream of very early events in the initiation of thymus/parathyroid organogenesis. Together, our findings are consistent with our previous observation that *Eya1* acts upstream of Six genes during early thymus and parathyroid development and provide insights into the molecular and developmental basis of thymus/parathyroid defects occurring in the *Eya1* or Six mutant mice.

Materials and methods

Animals and genotyping

Eya1;Six1 or *Pax1;Pax9* compound heterozygous mice were generated by crossing mice carrying mutant alleles of *Eya1* (Xu et al., 1999) and *Six1* (Laclef et al., 2003a,b) or *Pax1* (Wilm et al., 1998) and *Pax9* (Peters et al., 1998). *Six1; Six4* mice were generated as described (Grifone et al., 2005). Mouse strains used for experiments are 129/SvEv (*Eya1*, *Six1* single and *Eya1;Six1* double mutants) and C57BL/6J (*Six1* single, *Six1/Six4* double, *Pax1* single, *Pax9* single and *Pax1/Pax9* double mutants). Genotyping of mice and embryos was performed as previously described (Peters et al., 1998; Wilm et al., 1998; Xu et al., 1999, 2002).

Phenotype analyses and in situ hybridization

Embryos for histology and in situ hybridization were dissected out in PBS and fixed with 4% PFA at 4°C overnight. Embryonic membranes were saved in DNA isolation buffer for genotyping. Histology was performed as described (Xu et al., 1999). To visualize *Six1*^{lacZ}

expression, mutant embryos were stained with X-gal and sectioned as described (Xu et al., 2002).

For in situ hybridization, we used 6 wild-type or mutant embryos at each stage for each probe. Whole mount in situ was performed as described (Rosen and Beddington, 1993). For tissue section in situ hybridization, after fixing in 4% PFA overnight, embryos were dehydrated, embedded in wax and sectioned at 10 um. High-stringency hybridization, washing and RNase treatment were performed as described (Wilkinson and Green, 1990).

TUNEL analysis and antibody staining

We performed TUNEL assay for detecting apoptotic cell death using the ApopTag detection kit (Intergen). We used 6 wild-type or mutant embryos for this assay.

Section staining with calcitonin-specific antibody (ICN) was performed as described (Manley and Capecchi, 1995) and detected using HRP-coupled secondary antisera (Vector Laboratories) and staining with diaminobenzidine (DAB). Immunostained sections were counterstained with diluted Ehrlich's hematoxylin, cleared and mounted in Permount.

Results

Six1$^{-/-}$ mice lack the third pharyngeal pouch derivatives

Previous work described that *Six1*$^{-/-}$ mice lack thymus at E18.5, the only stage examined by sagittal sections. Here, we present a more detailed analysis of the morphological, molecular and genetic consequences for the thymus/parathyroid development in the absence of *Six1*. Transverse sections of the neck and upper trunk region of *Six1*$^{-/-}$ embryos from E14.5 to P0 revealed that the thymus and parathyroid, derivatives of the third pharyngeal pouches were absent in the mutant (asterisk, Figs. 1A, B, D, E). In situ hybridization and sections of X-gal-stained *Six1*^{lacZ} embryos at E14.5 revealed that *Six1* is expressed in the developing thymus and parathyroid (Figs. 1C, F and data not shown), suggesting that *Six1* plays a direct role in thymus and parathyroid development.

To determine the onset of phenotypic abnormalities during pharyngeal organ development, we analyzed *Six1*$^{-/-}$ embryos at E12.5. In wild-type embryos, the common primordia of thymus/parathyroid are morphologically apparent as epithelial buds at around E12.5 (Fig. 1G). In *Six1*$^{-/-}$ embryos, however, these structures were not observed (arrow, Fig. 1H). In contrast, the primordia of ultimobranchial bodies derived from 4th pouches appeared normal (Figs. 1G, H). *Eya1*, *Pax9* and *Pax1* are expressed in the primordia of thymus/parathyroid and ultimobranchial bodies at E12.5 (Figs. 1J, L and data not shown), whereas their expression was only visible in the rudiments of ultimobranchial bodies in *Six1*$^{-/-}$ embryos (Figs. 1K, M and data not shown). The lack of expression of these genes in the prospective anlage of the thymus/parathyroid primordia further confirms the absence of this structure in the mutant.

In contrast to *Eya1* and Pax genes, *Six1* expression was only observed in the thymus/parathyroid rudiments but not in the ultimobranchial bodies at E12.5 (Fig. 1I). In addition, no *Six1* expression was seen in the thyroid diverticulum or the developing thyroid lobe at any developmental stage (Fig. 1F and data not shown). These suggest that *Six1* may not play a direct role in thyroid morphogenesis. To determine this, we examined whether the *Six1* mutation also affects thyroid development. The thyroid is formed by the fusion of the thyroid diverticulum derived from the endoepithelium in the floor of the pharynx, and the ultimobranchial bodies (Hilfer, 1968; Rogers, 1927, 1971). In normal mice, the thyroid is a bilobed structure with two large lateral lobes joined across the ventral midline at their posterior aspects by the isthmus. Fig. 2A shows a transverse section through the lateral lobes

of a control animal and numerous calcitonin-producing parafollicular or C cells throughout the lobes as detected by an anticalcitonin antibody (Fig. 2B). In *Six1*$^{-/-}$ mice, the thyroid lobes appeared structurally normal but were hypoplastic (Fig. 2C). In contrast to the lack of fusion between the ultimobranchial body and the thyroid diverticulum observed in the *Eya1* mutant (Xu et al., 2002), these two structures were fused in *Six1*$^{-/-}$ animals and calcitonin-positive cells were seen throughout the lobes (Fig. 2D). However, the number of calcitonin-producing cells in the mutant was reduced to 49.5% of that in wild-type or heterozygous animals in all three animals analyzed (Fig. 2E). In addition, in all the cases examined, the thyroid lobe was reduced by approximately 36.5% and the follicular cells were also reduced in number (Figs. 2C, D and data not shown). Because *Six1* is expressed in the pharyngeal neural crest mesenchyme, the thyroid defect observed in the mutant is more likely due to lack of proliferative signals from neural crest cells expressing *Six1*.

Six1 and Six4 act synergistically to regulate the expression of Gcm2 and Foxn1

To further investigate the onset of developmental and molecular defects in the formation of thymus/parathyroid primordia in *Six1*$^{-/-}$ embryos, we first analyzed the expression of the parathyroid-specific marker *Gcm2* and the thymus-specific marker *Foxn1*. *Gcm2* is expressed in the third pharyngeal pouches at E10.5 (Fig. 3A), and its expression marks the prospective parathyroid anlage from E11.5 (Gordon et al., 2001). *Foxn1* regulates thymic epithelial cell differentiation (Blackburn et al., 1996; Su et al., 2003), and its expression is turned on in the common primordia from approximately E11.5 (Fig. 3D). In *Six1*$^{-/-}$ embryos, *Gcm2* expression in the third pouch was detectable at E10.5 but was largely reduced (Fig. 3B). Its expression disappeared completely by E11.5 (data not shown). *Foxn1* expression was also reduced in the common primordia of *Six1*$^{-/-}$ embryos at E11.5 (Fig. 3E). These results suggest that *Six1* is required for normal maintenance of *Gcm2* and *Foxn1* expression during early thymus/parathyroid development.

We have previously shown that no *Gcm2* and *Foxn1* expression was detected in *Eya1*$^{-/-}$ embryos (Xu et al., 2002). Therefore, the thymus/parathyroid organ defects appear to occur earlier in the *Eya1* mutant than in the *Six1* mutant. Since the closely related family member *Six4* is coexpressed with *Six1* in the pharyngeal endoderm from early stages and *Six4*-null mice appear to be normal (Ozaki et al., 2001), we hypothesized that these two genes may function redundantly in the pharyngeal endoderm. To test this, we first examined the developing third pouch from E9.5 to 11.5 by organ-specific marker gene analysis. In the double mutant, the expression of both *Gcm2* and *Foxn1* was completely absent at E10.5 and 11.5, respectively (Figs. 3C, F), suggesting that both *Six1* and *Six4* function synergistically to regulate *Gcm2* and *Foxn1* expression during early thymus/parathyroid formation.

Degeneration of the thymus/parathyroid rudiment in the absence of Six1 and Six4

The presence of *Foxn1* expression in E11.5 *Six1*$^{-/-}$embryos suggests that the third pouch is initially patterned into the organ primordia. Histological analyses of sagittal sections of E11.0 to 11.5 normal and mutant embryos confirmed that the third pouch is separated to form the rudiment for the thymus/parathyroid in *Six1*$^{-/-}$ embryos (Fig. 3H). However, the mutant primordia became morphologically smaller when compared with wild-type (Fig. 3G). This rudiment was also observed in the *Six1;Six4* double mutant (Fig. 3I) but appeared significantly smaller in size when compared to those in the single mutant (Fig. 3H). Because we failed to observe the thymus/parathyroid rudiments in E12.5 *Six1*$^{-/-}$ or *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos, it is possible that the rudiments observed in younger embryos degenerate and thus fail to develop further. We therefore sought to determine whether the endodermal cells in the developing thymus/parathyroid anlage undergo abnormal cell death in the mutants. *Six1*$^{-/-}$ mutant primordia exhibited increased cell death as demonstrated by TUNEL labeling of apoptotic nuclei (Fig. 3K), whereas very few apoptotic cells were seen in the controls (Fig.

3J). Elevated cell apoptosis was observed in the double mutant rudiments (Fig. 3L). Based on these data, we concluded that the defective formation of the thymus/parathyroid can be attributed, at least in part, to increased cell death.

We have also investigated whether the size reduction of thymus/parathyroid rudiments observed in the mutants at E11.0–11.5 is due to reduced cell proliferation by assaying BrdU-incorporation at E9.5 and 10.5, before apparent cell death was seen in the mutants. No obvious difference in the number of BrdU-labeled cells was detected between control and mutant embryos at these stages examined (data not shown), suggesting that *Six1* and *Six4* may not directly regulate cell proliferation of the pouch endoderm during early thymus/parathyroid organogenesis.

Pax1 expression in the endodermal pouches requires both Eya1 and Six1 functions

We have previously found that *Eya1* is required for the expression of *Six1* but not *Pax1*, *Pax9* and *Hoxa3* in pharyngeal pouch endoderm (Xu et al., 2002). To determine the regulatory relation between these genes, we first confirmed whether *Eya1* is upstream of Six genes. *Eya1* expression appeared to be normal in the *Six1* single (data not shown) or *Six1;Six4* double mutants by both whole mount and section in situ hybridization (Figs. 4A–D and data not shown), further confirming that *Eya1* is genetically upstream of the Six genes during early thymus/parathyroid organogenesis.

To further examine whether *Eya1* and *Six1* lie downstream of *Pax1/Pax9* genes during thymus/parathyroid development, we analyzed their expression in the *Pax9* single or *Pax1;Pax9* double mutant embryos. Interestingly, both *Eya1* and *Six1* expression in the third pouches appeared to be normal in either the *Pax9* single or the *Pax1;Pax9* double homozygous embryos (Figs. 4E–J), indicating that their expression is independent from *Pax1/Pax9*.

We next analyzed the expression of *Pax1*, *Pax9* and *Hoxa3* in the *Six1* single, *Six1;Six4* and *Eya1;Six1* double homozygous embryos to further clarify whether *Eya1*, *Six1*, *Pax1/Pax9* and *Hoxa3* genes function in the same regulatory pathway during early patterning of the third pharyngeal pouches. The expression of *Pax1*, normally detected in pharyngeal pouches in wild-type (Figs. 5A, D), *Six1*$^{-/-}$ (Figs. 5B, E) or *Six1*$^{-/-}$*; Six4*$^{-/-}$ (data not shown) embryos at E10.0, was undetectable in the *Eya1;Six1* double homozygous embryos by both section and whole mount in situ hybridization (Figs. 5C, F), whereas its expression in the somites appeared to be normal (Fig. 5F). *Pax9* expression was not detectably altered in the pharyngeal endoderm in the *Six1* single, *Six1;Six4* or *Eya1;Six1* double homozygous embryos (Figs. 5G–I and data not shown). It should be noted that the second pharyngeal arches are missing in the *Eya1;Six1* double homozygous embryos (Fig. 8). Previous studies have suggested that *Hoxa3* functions upstream of Pax genes, as *Pax1* expression was reduced in the *Hoxa3* mutant and these two genes genetically interact (Su et al., 2001). *Hoxa3* expression in the third pharyngeal endoderm and mesenchyme appeared to be unaffected in the *Pax1*$^{-/-}$;*Pax9*$^{-/-}$embryos at E10.0 (Figs. 5J, K), further suggesting that *Hoxa3* expression is *Pax1/Pax9*-independent. Its expression also appeared normal in *Six1*$^{-/-}$ or *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos (data not shown). In *Eya1*$^{-/-}$;*Six1*$^{-/-}$ embryos, *Hoxa3* expression was observed in the pharyngeal region at E9.5–10.5 (Fig. 6L). Together, these data clearly show that *Eya1*-Six is required for the expression of *Pax1* but not *Pax9* and *Hoxa3* in the entire pharyngeal endoderm.

Normal Tbx1, Fgf8 and Wnt5b expression in the third pouch endoderm requires Eya1 function

To better understand the developmental and molecular mechanisms by which *Eya1*-Six acts in the patterning of third pharyngeal pouches, we examined other genes that are known to be important for the formation of thymus/parathyroid. *Nkx2.6*, a member of the NK2 homeobox gene family homologous to the *Drosophila tinman* gene (Bodmer, 1993; Harvey, 1996; Tanaka et al., 1998), is expressed in the caudal pharyngeal pouches and is required for the development of the pharynx (Tanaka et al., 2001). At E9.75, *Nkx2.6* is expressed in the second, third and fourth pharyngeal pouches (Fig. 6A) and its expression levels appeared to be normal in *Six1*$^{-/-}$ or *Six1*$^{-/-}$;*Six4*$^{-/-}$ mutant embryos (Figs. 6B, C). *Tbx1* is required for the segmentation of the pharyngeal apparatus, and inactivation of *Tbx1* in mice causes thymic and parathyroid defects (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Vitelli et al., 2002). At E9.5, *Tbx1* is strongly expressed in the posterior third pharyngeal pouch and the mesodermal core of the pharyngeal arches (Fig. 6D; Vitelli et al., 2002), and its expression in the pharyngeal region appeared to be normal in *Six1*$^{-/-}$ embryos (Fig. 6E). However, its expression domain in the third pouch region appeared to be smaller in the double mutant (Fig. 6F) when compared with its normal expression. *Fgf8* has been shown to function downstream of *Tbx1* in the pharyngeal endoderm and both genes genetically interact during the differentiation of the pharyngeal arch arteries (Vitelli et al., 2002). *Fgf8* mutants also show absent or hypoplastic thymus and abnormal craniofacial development (Abu-Issa et al., 2002). At E9.5–10.5, *Fgf8* expression in the developing pharyngeal endoderm appeared to be normal in the *Six1* mutant (Figs. 6G, H) but also appeared to be reduced in the third pouch region in the double mutant embryos (Fig. 6I). The observation of reduced *Fgf8* expression in the pharyngeal region in the *Six1;Six4* double mutant is consistent with previous observation (Grifone et al., 2005). The alteration of *Tbx1* and *Fgf8* in the third pouch in the double mutant could be resulted from morphological alteration of the pouches, because the second and third pouches appeared to be smaller in the double mutant as labeled by *Eya1* expression (Figs. 4A, B). Nonetheless, these data suggest that activation of these genes in the pharyngeal endoderm is independent from *Six1* and *Six4*.

We next analyzed these markers in the *Eya1* mutant embryos to further understand the *Eya1*-Six regulatory mechanism. *Nkx2.6* expression appeared to be slightly reduced in the mutant third pouch (arrow in Figs. 7B and compare with A). Interestingly, *Tbx1* expression was reduced in the third pouch endoderm of E9.5 *Eya1*$^{-/-}$ embryos (arrow in Figs. 7D and compare with C), while its expression in the mesodermal core appeared to be normal. Sections of whole mount in situ embryos confirmed a clear reduction in the levels of *Tbx1* expression in the mutant pharyngeal endoderm (data not shown). *Fgf8* expression was also reduced in the third pharyngeal region (arrow in Figs. 7F, F′ and compare with E, E′). The wingled helix/forkhead box (Fox)-containing transcription factors have been shown to directly bind to *Tbx1* promoter and regulate *Tbx1* expression in the pharyngeal endoderm and head mesenchyme (Yamagishi et al., 2003). *Foxi3* is expressed in the pharyngeal pouch endoderm and surface ectoderm from very early stages (Fig. 7G; Ohyama and Groves, 2004) and its expression was unaffected in the *Eya1* mutant embryos (Fig. 7H), suggesting that *Foxi3* acts in a parallel pathway or upstream of *Eya1*. Recent studies have shown that Shh signaling is required for the expression of *Tbx1* and Fox genes in the pharyngeal endoderm and head mesenchyme (Yamagishi et al., 2003), and we have found that Shh expression in the pharyngeal endoderm is normal in *Eya1*$^{-/-}$ embryos (data not shown).

Secreted Wnt glycoproteins have been recently demonstrated to regulate *Foxn1* expression in both autocrine and paracrine fashions (Balciunaite et al., 2002). Wnt4 and Wnt5b have been previously shown to be expressed in the third pouches at E10.5 by RT-PCR (Balciunaite et al., 2002). As normal *Foxn1* expression also requires Six function, we tested

whether Six and *Eya1* genes might regulate Wnt-signaling molecules during early thymus organogenesis. By in situ hybridization, we failed to detect obvious *Wnt4* expression in pharyngeal pouches of E10.5 normal embryos (data not shown), whereas *Wnt5b* expression was detectable in second, third and fourth pouches from E10.0 (Figs. 7I, I′), consistent with previous observation obtained by RT-PCR (Balciunaite et al., 2002). *Wnt5b* expression was unaltered in *Six1*$^{-/-}$ and slightly reduced in *Six1*$^{-/-}$;*Six4*$^{-/-}$ double mutants (data not shown) but was undetectable in *Eya1*$^{-/-}$ pouches (Figs. 7J, J′), while its expression in the tail region was preserved in the *Eya1* mutant (Fig. 7J). This result suggests that Eya1 may regulate Wnt-signaling during early thymus organogenesis. Taken together, the presence of *Nkx2.6*, *Foxi3*, *Pax1*, *Pax9*, and *Hoxa3* (Xu et al., 2002) expression and significant changes in the expression of *Tbx1*, *Fgf8* and *Wnt5b* seen in E9.5–10.0 *Eya1* mutant embryos strongly suggest that this dysregulation is part of the mechanism that leads to the thymus/parathyroid organogenesis defects.

Malformation of the second pharyngeal arch in Six1;Six4 or Six1;Eya1 double mutants

Since the *Six1;Eya1* or *Six1;Six4* double mutant embryos exhibited malformation of the second pharyngeal arch by in situ hybridization (Figs. 3, 4 and 6), and both genes are also coexpressed in the pharyngeal arch mesenchyme, we performed whole mount in situ hybridization with molecular marker *Dlx* genes that are known to be important for pharyngeal arch development to confirm this phenotype (Fig. 8). Both *Dlx1* and *Dlx3* are expressed in the first and second pharyngeal arches (Fig. 8A and data not shown) and their expression was normal in the *Six1* or *Eya1* single homozygous embryos (Figs. 8B, C and data not shown). In *Eya1*$^{-/-}$; *Six1*$^{-/-}$ embryos, the first arch appeared to be normal at E10.5 as labeled by *Dlx1* and *Dlx3* but the second arch was completely absent (Fig. 8D and data not shown). In *Six1*$^{-/-}$; *Six4*$^{-/-}$ mutant embryos, the second arch was present but was severely hypoplastic and fused with the first arch as labeled by *Dlx3* expression and by histological analysis (arrow in Figs. 8E, F and compare with G). These results indicate that *Eya1*, *Six1* and *Six4* also control normal development of the second pharyngeal arches, consistent with the recent observation that the *Six2*, another member of the Six gene family, plays an important role in second arch development (Kutejova et al., 2005).

Discussion

Members of the Six family of proteins play a fundamental role in patterning the developing vertebrate skeletal muscle and auditory system; however, their precise cellular and developmental requirements in patterning the pharyngeal endoderm have not been investigated. In this study, we have demonstrated an essential role for *Six1* in the patterning of third pharyngeal pouches into organ primordia. Moreover, our data show that *Six4* may function synergistically with *Six1* during early thymus/parathyroid organogenesis.

The Eya-Six regulatory cassette is conserved during early thymus/parathyroid development

Early thymus development requires appropriate segmentation of the foregut endoderm and its subsequent interaction with mesenchyme. After endodermal segmentation, subsets of endodermal cells migrate laterally along the AP axis to form the pouches. Although *Eya1* and *Six1* are expressed early in the endoderm, they appear to be specifically involved in the patterning of a subset of endodermal cells into organ primordia rather than in playing a role in endoderm formation in general, since mutations in *Eya1* or *Six1* specifically affect the development of endodermal pouches but other endodermal tissues develop normally. The lack of *Pax1* expression in the *Eya1;Six1* double mutant pharyngeal pouches suggests that *Eya1*-Six may be required for pouch formation. Detailed analyses of pharyngeal pouch formation in the *Eya1*, *Six1* and *Six4* single and double mutants are underway in our

laboratory to clarify whether these genes also play an early role in pharyngeal pouch formation.

Although chick-quail chimera experiments have suggested that the endoderm provides the initiating signals for induction of the thymus (LeDouarin and Joterean, 1975), how the third pouch endoderm is induced to differentiate and acquire competence to establish the initial thymic/parathyroid rudiment remains largely unknown. Our studies have shown that, in the absence of *Eya1*, the patterning of the thymus- and parathyroid-specific domains from the third pouches is not initiated, because the thymic/parathyroid rudiments as well as *Gcm2* and *Foxn1* expression were undetectable (Xu et al., 2002). Thus, Eya1 may act as a determination factor for the initiation of thymus/parathyroid-specific developmental programs, and in the absence of Eya1, the third pouch endodermal cells fail to acquire competence for thymus/parathyroid development.

In contrast, in the *Six1* mutant, the patterning of the third pouch into thymus/parathyroid primordia is initiated but the organ-specific differentiation programs are blocked at early stages, as an alteration of *Gcm2* expression at E10.5–11.5 and *Foxn1* expression at E11.5 was observed. Therefore, the arrest in thymus and parathyroid development occurs earlier in the *Eya1* mutant than in the *Six1* mutant. This is consistent with the idea that *Six1* is downstream of *Eya1*. However, if *Six1* is a direct downstream target of *Eya1* and their gene products interact via protein–protein interactions to control other downstream gene expression, one might expect arrest in the *Six1* mutant mice to occur earlier, almost at the identical stages as in the *Eya1* mutant. Since the closely related family member *Six4* is coexpressed with *Six1* in the pharyngeal endoderm from early stages and *Six4* mutant mice are normal (Ozaki et al., 2001), *Six1* and *Six4* may function redundantly in the pouch endoderm during early thymus/parathyroid organogenesis with *Six1* alone executing later functions. This could explain why the *Six1* mutant phenotype occurs slightly later. If *Six1* and *Six4* function redundantly in early thymus/parathyroid organogenesis and a crucial threshold of *Six1*/*Six4* protein expression in pharyngeal pouches regulates other gene expression, their expression should be reduced in the double mutant embryos. In support of this, our results show a complete absence of *Gcm2* and *Foxn1* expression in the double mutant, suggesting that the patterning of the third pouch into the thymus/parathyroid-specific domains is not initiated. Therefore, the threshold of *Six1*/*Six4* appears to be crucial for the regulation of the organ primordia-specific gene expression. The presence of hypoplastic rudiments derived from the third pouches in the *Six1;Six4* double mutant but not in the *Eya1* mutant is consistent with the idea that *Eya1* is upstream of Six genes. Together, these results indicate that *Six1* and *Six4* act downstream of *Eya1* to synergistically control morphogenetic movements of early thymus/parathyroid tissues, representing at some level a degree of functional redundancy.

Regulatory relation between Eya1-Six, Pax and Hox genes in the pharyngeal region

The developmental pathways responsible for the thymus/parathyroid formation have remained rather obscure. Recent genetic studies have suggested that a *Hoxa3-Pax1/Pax9-Eya1-Six1* regulatory pathway may be operating during thymus/parathyroid organogenesis based on two lines of evidence. First, *Pax1* expression was reduced in the *Hoxa3* mutant pharyngeal pouches and it also genetically interacts with *Hoxa3* during thymus development (Dietrich and Gruss, 1995; Wallin et al., 1996; Manley, 2000; Su et al., 2001). Second, *Six1* expression but not *Pax1*, *Pax9* and *Hoxa3* was lost in $Eya1^{-/-}$ pharyngeal endoderm (Xu et al., 2002). In this study, we show normal expression of *Hoxa3*, *Eya1* and *Six1* in the pharyngeal pouches in the *Pax9* single or the *Pax1;Pax9* double mutant embryos. In contrast, *Pax1* expression in the entire pharyngeal endoderm was completely lost in the *Eya1;Six1* double mutant embryos at E9.5–10.5, indicating that *Pax1* is downstream of

Eya1-Six in pharyngeal endoderm. Our data suggest that *Pax9* may function in parallel with or independent from *Eya1-Six* in the pharyngeal endoderm as it is also expressed in the *Eya1;Six1* double mutant.

In contrast to the expression of Pax, *Eya1* and Six genes in the entire pharyngeal endoderm from early stages, *Hoxa3* expression is restricted to the third and fourth pharyngeal region. This suggests that the *Hoxa3* may be required for specifying the identity of pharyngeal organs that develop from the third and fourth pharyngeal endoderm but is dispensable for pouch formation, while *Eya1*, Six and Pax genes may have an early role in the formation of pharyngeal pouches. In support of this view, we have found that *Hoxa3* is expressed normally in the third pharyngeal endoderm of *Eya1* single, *Eya1;Six1*, *Six1; Six4* and *Pax1;Pax9* double mutants. Although the expression of *Eya1* in the *Hoxa3* mutant remains to be analyzed, it is possible that *Eya1*, *Six1* and *Six4* act downstream of *Hoxa3* in a genetic cascade leading to the initiation of the parathyroid and thymus differentiation program by activating *Gcm2* and *Foxn1* as their expression requires the function of *Eya1*, *Six1/Six4* and *Hoxa3* (Fig. 9).

Genetic analyses have shown that the thymus/parathyroid phenotype is strikingly similar between the *Eya1* and *Hoxa3* mutant mice (Manley and Capecchi, 1995; Xu et al., 2002). In addition to the third pouch endoderm, *Eya1* and *Six1* are coexpressed with *Hoxa3* and *Hoxb3* in neural crest-derived arch mesenchyme and ectoderm, respectively (Manley and Capecchi, 1995, 1998; Xu et al., 2002), further suggesting possible interaction between these genes in all three tissue layers. In support of this view, recent studies have raised the possibility that Eya and Hox genes may act together to regulate Six gene expression in different developmental systems (Wellik et al., 2002; Kutejova et al., 2005). During metanephric induction, *Six2* expression in the mesenchyme was undetectable in the *Hoxa11;Hoxc11;Hoxd11* triple mutant or the *Eya1* mutant, whereas *Eya1* expression was normal in the *Hox11* triple mutant (Wellik et al., 2002). This suggests that *Eya1* and *Hox11* genes may function in parallel to regulate Six gene expression. Recent data from our laboratory suggest that *Eya1* may function upstream of *Hox11* genes because the metanephric defect appears to occur earlier in the *Eya1* mutant than in the *Hox11* triple mutant (Sajithlal et al., 2005). Detailed expression studies of Hox, Eya and Six genes in wild-type and respective mutant embryos should help to clarify the regulatory relation between these genes in the pharyngeal region.

Regulatory relation between Eya1, Six and Tbx1-Fgf8 in the pharyngeal endoderm

Our data clearly show that *Eya1* appears to be upstream of very early events in the initiation of thymus/parathyroid organogenesis. This is further supported by downregulation of *Tbx1* and *Fgf8* expression in *Eya1* or *Six* mutant thymus/parathyroid development. First, the expression level of *Tbx1* was severely reduced in the third pharyngeal pouch region in $Eya1^{-/-}$ embryos. Along with the reduced *Tbx1* expression, we also found decreased *Fgf8* expression in the pharyngeal endoderm. The reduced *Fgf8* expression in the mutant could be the result of the downregulation of endodermal *Tbx1* expression, because *Tbx1* has been shown to regulate *Fgf8* expression in the pharyngeal endoderm (Vitelli et al., 2002) and both genes are required for normal thymus/parathyroid development (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Abu-Issa et al., 2002). Thus, Eya1 appears to regulate the expression of *Tbx1-Fgf8* in the pharyngeal pouches. As a reduction of *Fgf8* expression was also observed in the *Six1;Six4* double homozygous embryos (Fig. 7 of this manuscript; Grifone et al., 2005), Six proteins may also be involved in this regulation (Fig. 9).

Previous data (Yamagishi et al., 2003) support a model in which *Tbx1* is directly regulated by different Fox proteins in the pharyngeal endoderm and head mesenchyme through a

common-Fox-binding site upstream of *Tbx1*. Shh signaling functions to maintain the expression of different *Fox* genes, which subsequently activate *Tbx1* expression. We have found that *Foxi3*, *Foxa2* and *Shh* expression was unaffected in *Eya1*$^{-/-}$ embryos (Fig. 7 and data not shown), suggesting that the expression of *Shh*, *Foxi3* and *Foxa2* in the pharyngeal endoderm is independent from *Eya1*. Since Eya1 cannot bind to DNA directly, Eya1 may act as a mediator of Shh signaling and interact with Fox proteins to regulate *Tbx1* expression in the endoderm. This hypothesis is currently being tested in our laboratory.

In summary, our results show that mutations in both *Eya1* and *Six1*, the Branchio-Oto-Renal syndrome causing genes in humans, critically affect pharyngeal morphogenesis, thus providing insights into molecular and developmental mechanisms governing early thymus/parathyroid organogenesis.

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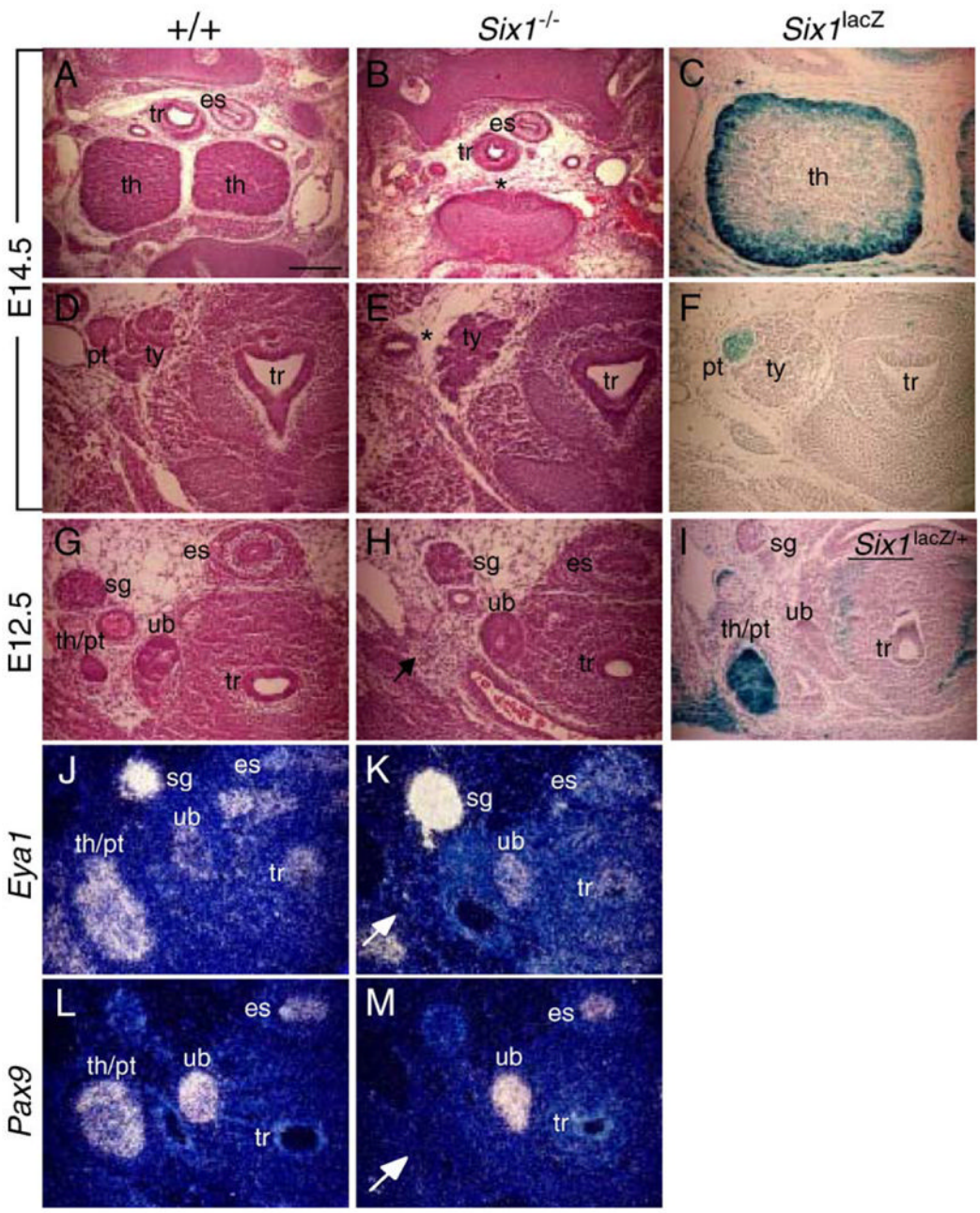

Fig. 1.

Six1$^{-/-}$ embryos lack thymus and parathyroid glands. (A, B, D, E, G, H) H&E-stained transverse sections of the neck region at E14.5 and 12.5. (A, D) In wild-type embryos, two thymic lobes (th) are present and the parathyroid glands (pt) are associated with the thyroid gland (ty) at E14.5. (G) At around E12.5, the 3rd pouch-derived thymus (th)/parathyroid (pt) primordia and the 4th pouch-derived ultimobranchial bodies (ub) in wild-type embryos are evident. (B, E, H) In *Six1*$^{-/-}$ embryos, no thymus and parathyroid formation (*) was found at the same level or in other regions of the neck and upper trunk at E14.5 (B, E). At E12.5, in *Six1*$^{-/-}$ embryos, the primordia of thymus/parathyroid from the 3rd pouches failed to form (arrow in panel H), while the rudiments of ultimobranchial bodies (ub) derived from 4th pouches are present. (C, F, I) Transverse sections showing *Six1* expression in the thymic

lobes (th) and parathyroid glands (pt) at E14.5 and in the thymus/parathyroid primordia at E12.5 by X-gal staining for *Six1*^{lacZ}. (J–M) Radioisotope in situ hybridization of transverse sections showing that *Eya1* and *Pax9* are normally expressed in the rudiments of the thymus/parathyroid and ultimobranchial bodies at E12.5 (J, L); (K, M) however, *Six1*$^{-/-}$ embryos show absence of these genes' expression in the thymus/parathyroid rudiments, further indicating the absence of these structures in the mutant. Their expression was unaffected in the rudiments of ultimobranchial bodies in the mutant. For all panels, dorsal is up. Other abbreviations: es, esophagus; tr: trachea. Scale bars: A, B, 100 μm; C–M, 50 μm.

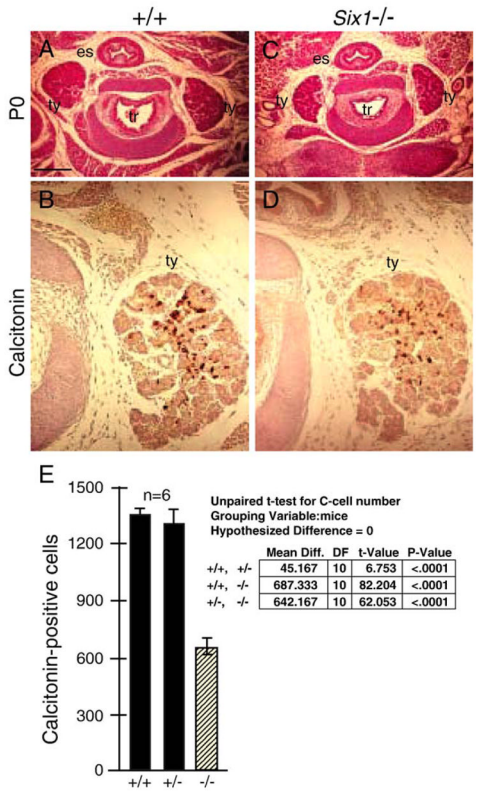

Fig. 2.

Thyroid lobes appear to be smaller in *Six1*$^{-/-}$ mice. (A, B) H&E-stained transverse section through newborns showing the bilobed thyroid (ty) in wild-type animals (A) and numerous calcitonin-positive cells are throughout the lobe stained with anticalcitonin antibody (brown staining, B). (C, D) In *Six1*$^{-/-}$ animals, the thyroid lobes appear to be smaller but calcitonin-producing cells are throughout the lobes. (E) The number of Calcitonin-positive cells was counted from each thyroid lobe, and 6 lobes for each genotype were counted and the numbers were averaged. Data refer to the average of six thyroid lobs (three embryos) per genotype; *P* values were calculated using StatView *t* test. Error bars indicate standard deviation. Dorsal is up. Scale bars: A, C, 100 μm; B, D, 30 μm.

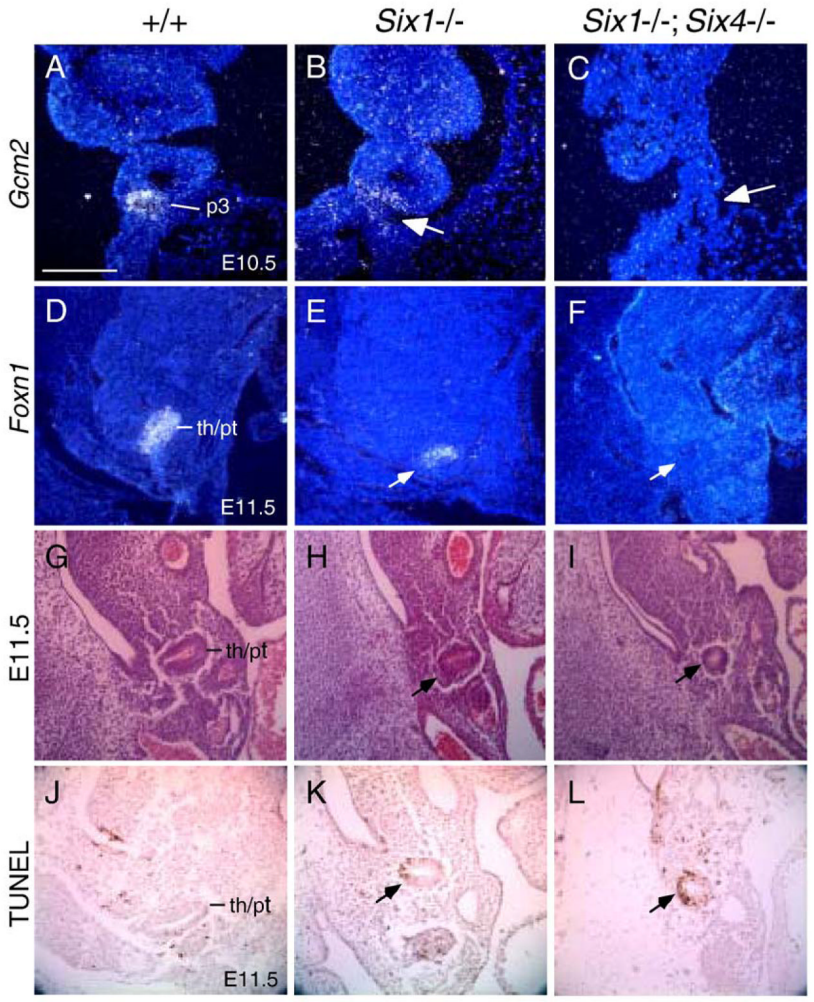

Fig. 3.

Six1 and *Six4* act synergistically to regulate early thymus/parathyroid organogenesis. (A–C) *Gcm2* is normally expressed in the 3rd pouch endoderm (p3) at E10.5 (A); however, its expression was reduced in *Six1*$^{-/-}$ embryos at E10.5 (arrow, B) and was undetectable in *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos (arrow, C). (D–F) *Foxn1* is expressed in the common primordia (th/pt) at E11.5 in normal embryos (D). Its expression was reduced in *Six1*$^{-/-}$ embryos (arrow, E) and was absent in *Six1*$^{-/-}$;*Six4*$^{-/-}$embryos (arrow, F). (G–I) H&E-stained sagittal sections show the developing thymus/parathyroid rudiments that are separated from the pharynx in normal embryos at E11.5 (G). In *Six1*$^{-/-}$ (H) or *Six1*$^{-/-}$;*Six4*$^{-/-}$ (I), the rudiments do separate from the pharynx but appeared to be slightly smaller in the single mutant (arrow, H) and significantly smaller in the double mutant (arrow, I). (J–L) TUNEL assay for detecting apoptotic cells. (J) In normal embryos, only a few apoptotic cells were seen in the common primordia (th/pt), (K) whereas increased apoptotic cells were detected in *Six1*$^{-/-}$ thymus/parathyroid primordia (arrow). (L) In *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos, cell apoptosis was elevated (arrow). (M) Statistic analysis of apoptotic cells. Data (Mean) refer to the average of six

primordia (three embryos) per genotype; *P* values were calculated using StatView *t* test. Panels A–C are coronal sections and all others are sagittal sections. For all panels, anterior is up. For panels D–L, dorsal is to the left. Scale bars: 100 μm for all panels.

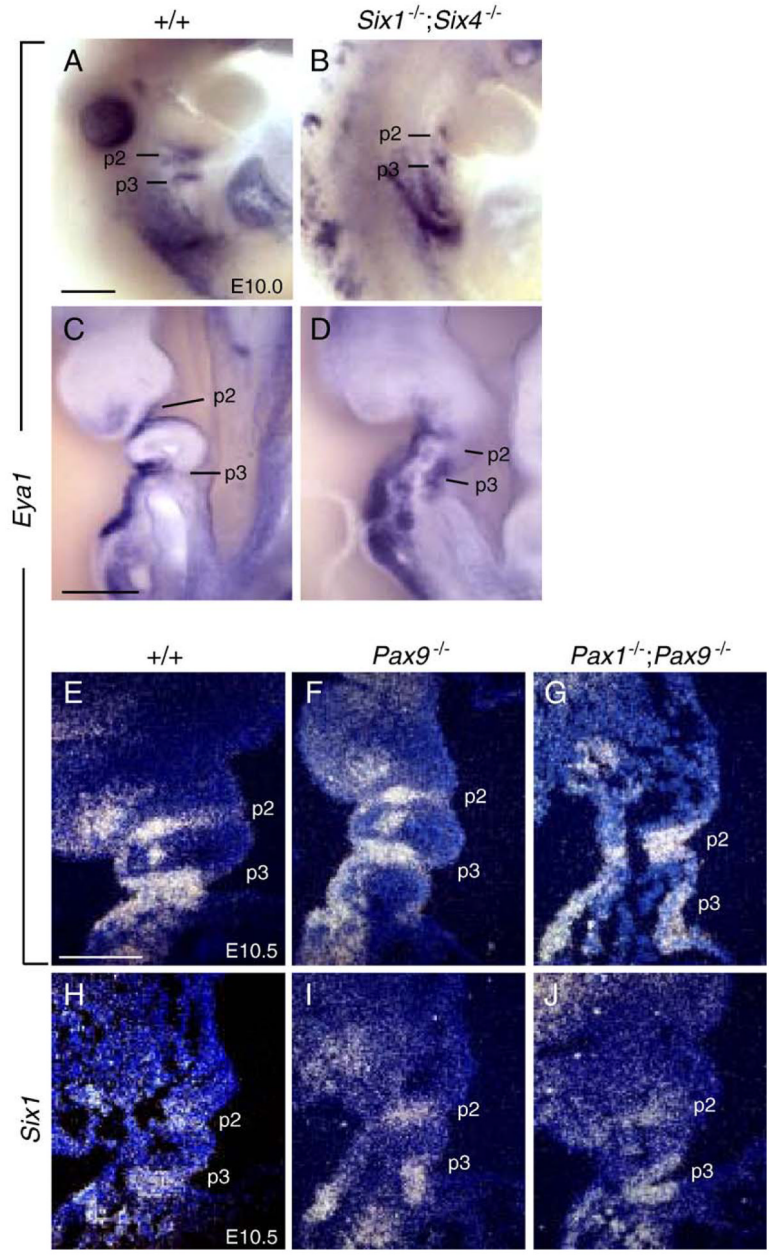

Fig. 4.

Eya1 is upstream of Six genes and both *Eya1* and *Six1* expression in the pharyngeal endoderm is *Pax1/Pax9*-independent. (A, B) Lateral view of whole mount in situ embryos showing *Eya1* expression in the pharyngeal pouches (p2, p3) in normal (A) and $Six1^{-/-};Six4^{-/-}$ double mutant embryos (B). (C, D) Coronal sections of whole mount in situ embryos showing that *Eya1* expression level is similar between normal (C) and *Six1;Six4* double mutant (D) embryos at E10.0. (E–G) Section in situ hybridization showing *Eya1* expression in normal embryos in the pharyngeal pouches (p2, p3) at E10.5. Its expression was unaltered in $Pax9^{-/-}$ (F) or $Pax1^{-/-}; Pax9^{-/-}$ (G) embryos. (H–J) Similarly, *Six1* is expressed in the pharyngeal pouches at E10.5 (H) and its expression appeared to be unaffected in $Pax9^{-/-}$ (I) or $Pax1^{-/-}; Pax9^{-/-}$ (J) embryos. For all panels, anterior is up. Scale bars: 100 μm.

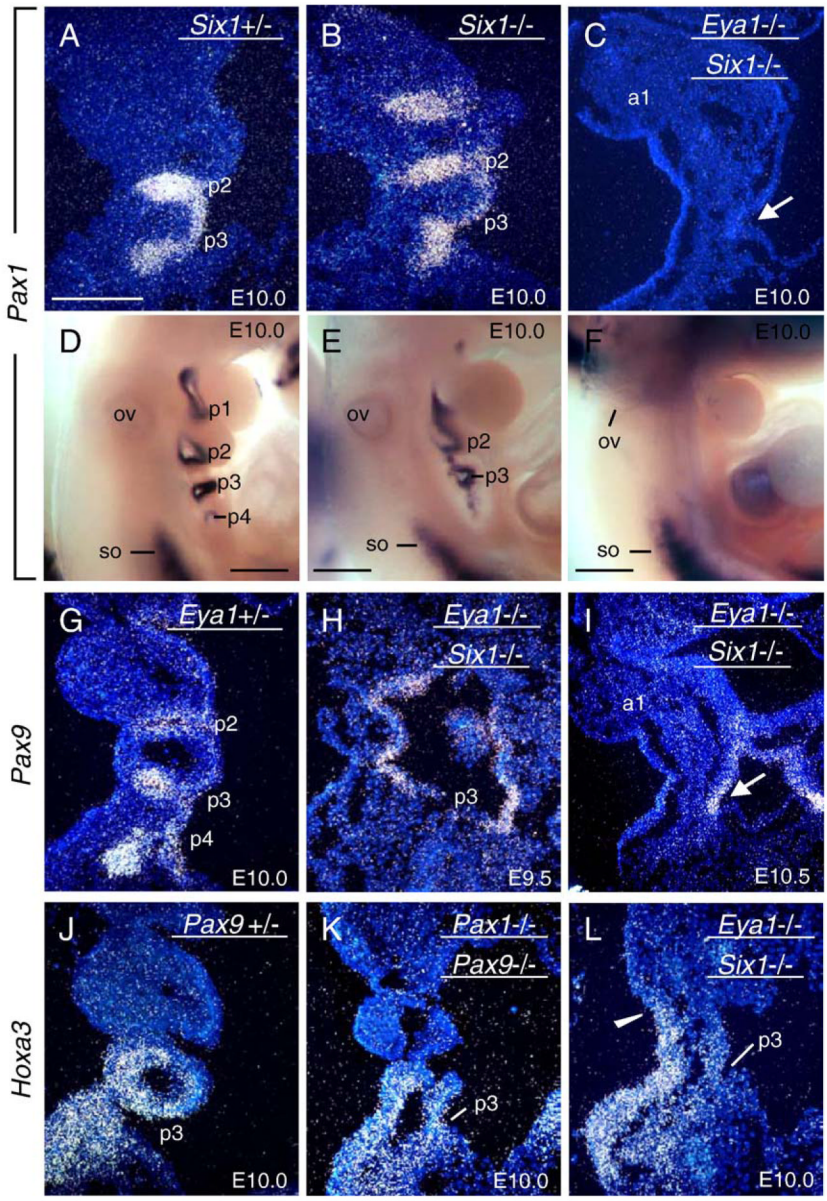

Fig. 5.

Pax1 expression is markedly reduced in the *Eya1;Six1* double homozygous embryos. (A–F) Section (A–C) and whole mount (D–F) in situ hybridization showing that *Pax1* is expressed in all pouches at E10.0 (A, D) and its expression was normal in $Six1^{-/-}$ pouches (B, E); however, its expression was undetectable in $Eya1^{-/-};Six1^{-/-}$ embryos (arrow, C, F). (G–I) *Pax9* is expressed in all pouches (p2–p4) in control embryos at E9.5–10.5 (G), and (H, I) its expression was observed in the pharyngeal endoderm of $Eya1^{-/-};Six1^{-/-}$ double homozygous embryos at these stages with a slight reduction at E10.5 (arrow, I). (J–L) *Hoxa3* is expressed in the 3rd and 4th pharyngeal pouches and arches (a3) in control embryos at E10.0 (J) and its expression was normal in $Pax1^{-/-};Pax9^{-/-}$ embryos (K). (L) In $Eya1^{-/-};Six1^{-/-}$double homozygous embryos, *Hoxa3* is expressed in the third pharyngeal region with a slight reduction in the endoderm only. Arrowhead points to the anterior limit of the 3rd arch in the *Eya1;Six1* double homozygotes. Note that *Eya1;Six1* double

homozygotes lack the 2nd pharyngeal arch. For all panels, anterior is up. Scale bars: 100 μm.

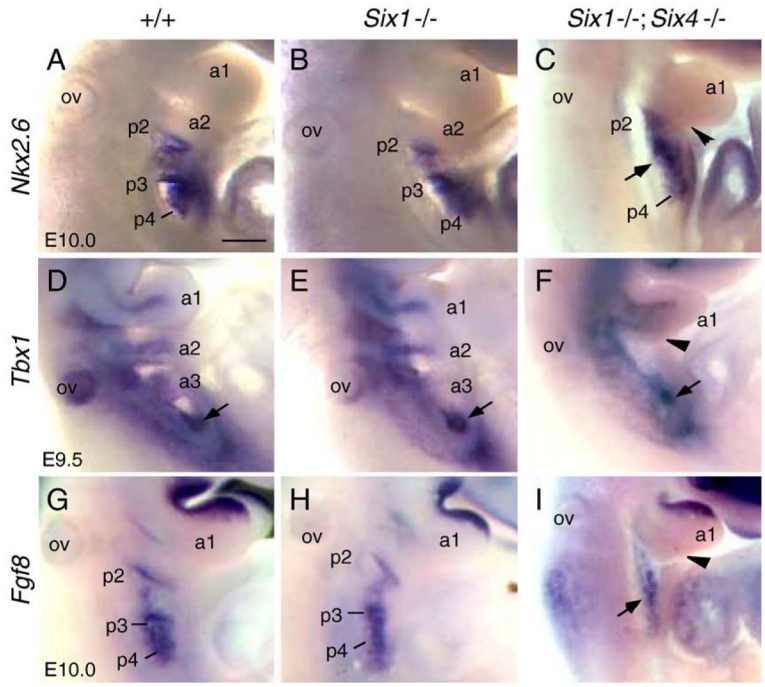

Fig. 6.

Marker gene analysis in *Six1*$^{-/-}$ and *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos. (A–C) *Nkx2.6* is expressed in the 2nd–4th pouches (p2–p4) in wild-type embryos at E10.0 (A) and its expression level was normal in *Six1*$^{-/-}$ (B) or *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos (C). (D–F) *Tbx1* is strongly expressed in the posterior region of the 3rd pouch in wild-type embryos at E9.5 (arrow, D) and in the mesodermal core of the arches (a1–a3), and its expression in these structures was unaffected in *Six1*$^{-/-}$ embryos (E); (F) however, its expression in these structure was reduced in *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos. Arrow points to the 3rd pouch region and arrowhead points to the 2nd arch, which is hypoplastic and fused with the 1st arch. (G–I) *Fgf8* is expressed in the pharyngeal pouches at E10.0 (G) and its expression was normal in *Six1*$^{-/-}$ embryos (H); however, (I) its expression in the 3rd pouch region appeared to be reduced in *Six1*$^{-/-}$;*Six4*$^{-/-}$ embryos (arrow). For all panels, anterior is up and dorsal is to the left. Scale bars: 100 μm.

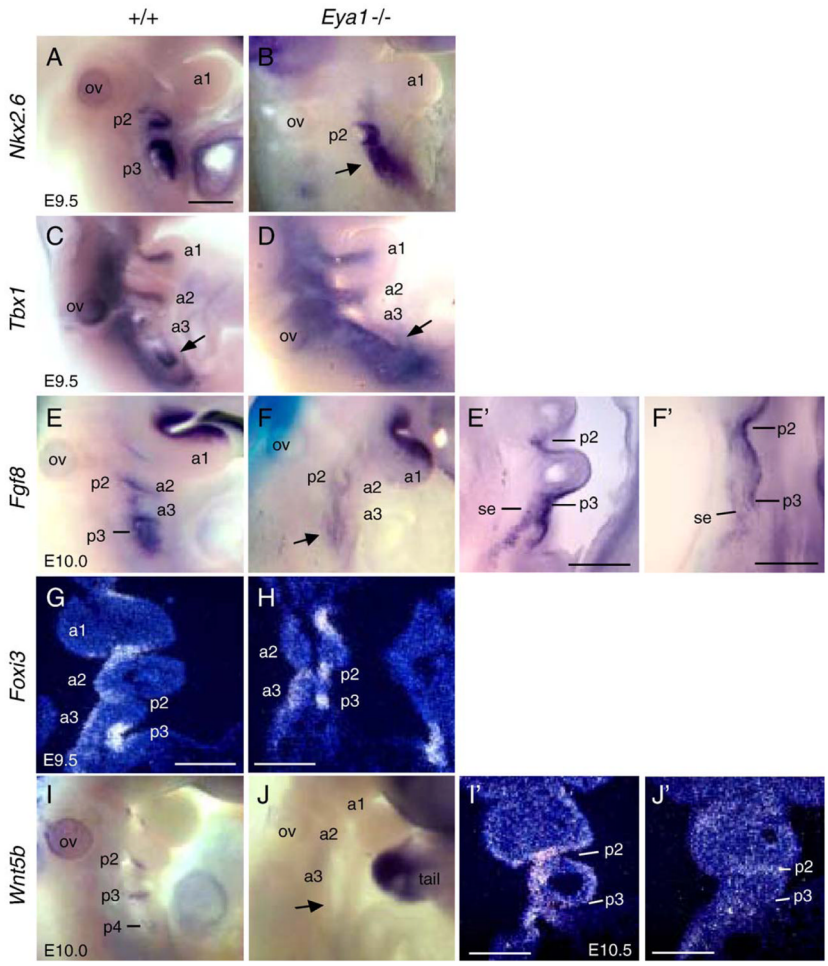

Fig. 7.

Eya1 is required for normal expression of *Tbx1*, *Fgf8* and *Wnt5b*. (A, B) Whole mount in situ showing that *Nkx2.6* is expressed in the pharyngeal pouches at E9.5 (A), and (B) that its expression levels appear to be slightly reduced in *Eya1*$^{-/-}$ embryos (arrow). (C, D) Whole mount in situ showing that *Tbx1* is strongly expressed in the posterior 3rd pouch endoderm (arrow, C), and (D) that its expression in the 3rd pouch was markedly reduced in *Eya1*$^{-/-}$ embryos (arrow); however, its expression in the mesodermal core of the pharyngeal arches was not detectably altered in the mutant. (E, F) Whole mount in situ showing normal *Fgf8* expression in the pouch endoderm (E) and its reduced expression in the Eya1 mutant (arrow, F). (E′, F′) Sections of whole mount embryos confirmed a reduction of *Fgf8* expression in the third pharyngeal endoderm and ectoderm (se). (G, H) Coronal sections showing that *Foxi3* is expressed in the pharyngeal ectoderm and endoderm from early stages (G), and that its expression appeared to be normal in *Eya1*$^{-/-}$ embryos (H). (I, J) Whole mount in situ showing that *Wnt5b* is expressed in the 2nd–4th pouches from E10.0 (I) and its expression was undetectable in *Eya1*$^{-/-}$ embryos (arrow, J). In contrast, its expression in the tail was normal in the mutant. (I′, J′) Section in situ confirmed that *Wnt5b* is expressed in the pharyngeal endoderm in normal embryos (I′) and its expression is absent in *Eya1*$^{-/-}$ embryos (J′) at E10.5. Other abbreviation: ov, otic vesicle. For all panels, anterior is up. Scale bars: 100 μm.

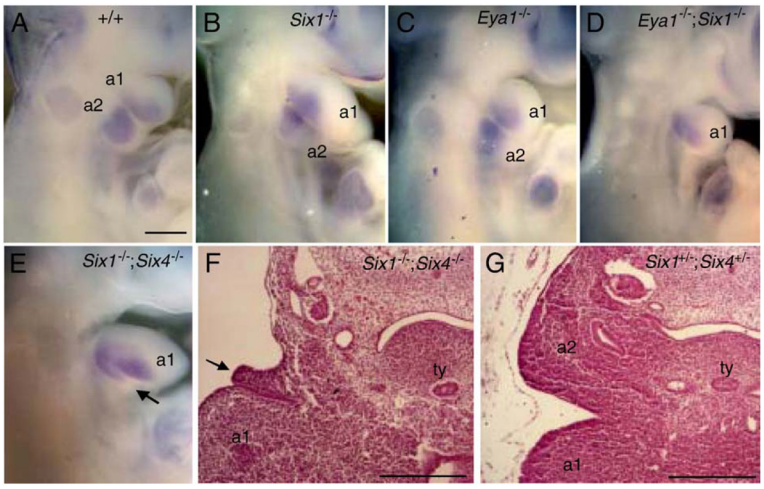

Fig. 8.

Malformation of the second arch in the *Eya1;Six1* or *Six1;Six4* double mutant. (A–E) Whole mount in situ showing *Dlx3* expression in the 1st and 2nd arches in E10.5 normal embryo (A). (B, C) Its expression is not detectably altered in the *Six1* (B) or *Eya1* single mutants (C). (D) However, in the *Eya1;Six1* double mutant, its expression was only visible in the 1st arch, and 2nd arch is absent in the double mutant. (E) In the *Six1;Six4* double mutant, the 2nd arch is hypoplastic and fused with the 1st arch as labeled by *Dlx3* expression (arrow). (F, G) H&E-stained transverse sections further confirmed the fusion between the 1st and 2nd arches in the *Six1;Six4* double mutant. In addition, the 2nd arch is hypoplastic (arrow, F). Other abbreviation: ty, thyroid. For panels A–E, anterior is up and dorsal is to the left. For panels F and G, dorsal is up. Scale bars: 100 μm.

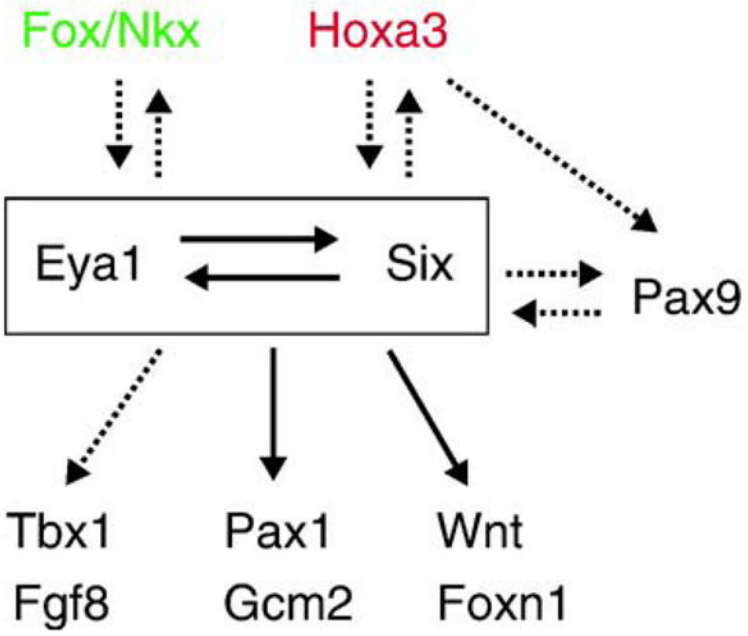

Fig. 9.

A hypothetical model for the Eya1-Six regulation during pharyngeal pouch development and thymus/parathyroid organogenesis. Our data clearly show that Eya1 is upstream of Six genes in the pharyngeal endoderm. It is known that Eya1 and Six proteins also physically interact (Ohto et al., 1999; Buller et al., 2001). Our data support a model in which *Pax1* is regulated by Eya1-Six in all pharyngeal pouches. *Tbx1* and *Fgf8* are also involved in pharyngeal pouch formation as well as thymus organogenesis (Hu et al., 2004; Xu et al., 2005), and their expression may be regulated by Eya1-Six. The Fox and Nkx genes are involved in endoderm formation and our data suggest that they may function upstream of or in parallel with Eya1-Six. During the initiation of thymus/parathyroid organogenesis, *Hoxa3* may function upstream or in parallel with Eya1-Six to regulate the expression of organ-specific genes *Gcm2* and *Foxn1* as well as other patterning genes. Our data also show that *Pax9* may function upstream of or in parallel with Eya1-Six during thymus/parathyroid organogenesis.