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Eya1 acts as a critical regulator for specifying the metanephric mesenchyme

Gangadharan Sajithlal, Dan Zou, Derek Silvius, Pin-Xian Xu*

McLaughlin Research Institute for Biomedical Sciences, Great Falls, MT 59405, USA

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Abstract

Although it is well established that the Gdnf-Ret signal transduction pathway initiates metanephric induction, no single regulator has yet been identified to specify the metanephric mesenchyme or blastema within the intermediate mesoderm, the earliest step of metanephric kidney development and the molecular mechanisms controlling *Gdnf* expression are essentially unknown. Previous studies have shown that a loss of Eya1 function leads to renal agenesis that is a likely result of failure of metanephric induction. The studies presented here demonstrate that Eya1 specifies the metanephric blastema within the intermediate mesoderm at the caudal end of the nephrogenic cord. In contrast to its specific roles in metanephric development, Eya1 appears dispensable for the formation of nephric duct and mesonephric tubules. Using a combination of null and hypomorphic *Eya1* mutants, we now demonstrated that approximately 20% of normal Eya1 protein level is sufficient for establishing the metanephric blastema and inducing the ureteric bud formation but not for its normal branching. Using *Eya1*, *Gdnf*, *Six1* and *Pax2* mutant mice, we show that *Eya1* probably functions at the top of the genetic hierarchy controlling kidney organogenesis and it acts in combination with *Six1* and *Pax2* to regulate *Gdnf* expression during UB outgrowth and branching. These findings uncover an essential function for Eya1 as a critical determination factor in acquiring metanephric fate within the intermediate mesoderm and as a key regulator of *Gdnf* expression during ureteric induction and branching morphogenesis.
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Introduction

In mammals, kidney development occurs in three stages that are all characterized by the mesenchymal-to-epithelial transformation within the intermediate mesoderm. The development of pronephros, the first kidney, is initiated at embryonic day 8 (E8) in mice by signals from the somite and surface ectoderm that induce cells in the intermediate mesoderm of the anterior region of the embryos to differentiate into nephric (Wolffian) duct and tubules (Obara-Ishihara et al., 1999; Mauch et al., 2000). The mesonephros appears at E9.5 when the Wolffian duct extends caudally towards the cloaca and induces the adjacent nephrogenic mesoderm to aggregate and form mesonephric tubules. Both the pro- and mesonephros regress shortly after their formation. The development of metanephros, the permanent kidney, initiates at approximately E10.5 when the ureteric bud (UB) appears as a thickening of the Wolffian duct at the level of the posterior half of the hindlimb (Grobstein, 1953; Saxen, 1987). Its development involves several distinct processes: first, establishment of the metanephric mesenchyme or blastema from intermediate mesoderm; second, induction and outgrowth of the UB; third, branching of the UB and differentiation of the metanephric mesenchyme to renal epithelial cells. The metanephric blastema appears as an aggregate of cells at the caudal end of the nephrogenic cord at around E10.5 in mice. It seems that the creation of metanephric blastema and the development of metanephros depend on the proper formation of the Wolffian duct during pronephros induction and its normal elongation.

* Corresponding author. Fax: +1 406 454 6019.
E-mail address: pxu@po.mri.montana.edu (P.-X. Xu).

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The homeodomain protein Lim1, which is expressed early in the intermediate mesoderm, the Wolffian duct and pro- and mesonephric tubules (Fujii et al., 1994), is required for all kidneys (Tsang et al., 2000). In *Lim1*$^{-/-}$ mice, the intermediate mesoderm is disorganized and fails to express other proteins necessary for kidney development (Tsang et al., 2000). The paired-domain proteins Pax2 and Pax8 are also expressed in the Wolffian duct and pro- and mesonephric tubules and the *Pax2*$^{-/-}$; *Pax8*$^{-/-}$ mice exhibit a complete absence of pro-, meso- and metanephric development (Bouchard et al., 2002). However, except *Lim1* and *Pax2;Pax8* double mutant mice, deletion of all other regulators of kidney organogenesis published so far all showed a morphologically apparent metanephric blastema. Therefore, the actual regulators that specify the metanephric blastema before metanephric induction are unidentified.

Subsequent to the formation of metanephric blastema, metanephric induction between the blastema and the Wolffian duct occurs to initiate UB development. Targeted mutagenesis has demonstrated that the establishment of a functional metanephric mesenchyme is a central step in kidney organogenesis as the Glial cell line-derived neurotrophic factor (Gdnf) secreted from the mesenchyme has been shown to promote ureteric development (reviewed by Vainio and Lin, 2002; Davies and Fisher, 2002). Gdnf acts as a mesenchyme-derived ligand that binds to its receptor tyrosine kinase (Ret) and Gfrα1 coreceptor, both of which are expressed in the Wolffian duct, and induces a UB (Durbec et al., 1996; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1996; Sainio et al., 1997; Cacalano et al., 1998; Enomoto et al., 1998; Sariola and Saarma, 1999). However, on its own, Gdnf fails to promote proliferation and branching morphogenesis of the isolated UB (Qiao et al., 1999). Pax2 has been suggested to regulate the transcription of *Gdnf* (Brophy et al., 2001). *Pax2* is expressed in both the mesenchyme and the ureteric epithelium. In *Pax2*$^{-/-}$ mice, although the metanephric blastema is clearly specified, it is incompetent for tubulogenesis (Torres et al., 1995; Brophy et al., 2001). Several other mesenchymal factors have also been shown to play a role in mediating the competence of the mesenchyme for UB outgrowth (Nishinakamura et al., 2001; Xu et al., 2003). However, despite the identification of these molecules as important regulators of kidney organogenesis by controlling the competence of the mesenchyme for UB development, whether these factors interact with each other to coordinate the complex pathways involved in kidney organogenesis and the molecular mechanisms controlling *Gdnf* expression is not established.

We have previously reported that *Eya1*, a homolog of *Drosophila eyes absent* (*eya*) gene, is expressed in the mesenchyme and deletion of this gene in mice leads to renal agenesis (Xu et al., 1999). Haploinsufficiency for the human *EYA1* gene results in Branchio-Oto-Renal (BOR) syndrome, an autosomal dominant disorder with incomplete penetrance and variable expressivity characterized by combinations of branchial, otic and renal anomalies (Abdelhak et al., 1997a,b; Kumar et al., 1998). *Eya1* gene encodes a transcription coactivator containing a divergent N-terminal activation domain and a conserved C-terminal Eya domain that mediates protein–protein interactions with Sine oculis and Dachshund proteins (Xu et al., 1997a,b; Chen et al., 1997; Pignoni et al., 1997). In *Drosophila*, *eya* functions in a molecular network with the fly *Pax6* gene *eyeless* (*ey*), *sine oculis* (*so*) and *dachshund* (*dach*) to regulate eye morphogenesis (reviewed in Treisman, 1999). The components of this network have been highly conserved during evolution with related genes in mammals regulating the development of multiple organ systems (Xu et al., 2003; Zheng et al., 2003). In early mammalian kidney development, the *Six1*, a member of the Six gene family homologous to *Drosophila so*, is transiently expressed in the metanephric mesenchyme before and after induction and loss of *Six1* leads to an incomplete invasion of the UB into the mesenchyme and incompetence of the mesenchyme for tubulogenesis (Xu et al., 2003). Surprisingly, the expression of *Pax2* and *Six2*, another member of the Six gene family, in the mesenchyme depends on Six1 function (Xu et al., 2003). Interestingly, we have found that Eya1 interacts with Six1 during kidney and auditory system development (Xu et al., 2003; Zheng et al., 2003) and mutations in the human *SIX1* also cause BOR syndrome (Ruf et al., 2004). Previous studies have shown that Eya1 may regulate the expression of *Gdnf*, *Six1* and *Pax2* (Xu et al., 1999, 2003). However, the identity of the steps at which Eya1 functions in early kidney morphogenesis and the developmental and molecular basis for renal defects observed in *Eya1*-deficient mice or BOR syndrome are unclear.

The studies presented here provide new insights into the role of Eya1 in the formation of metanephric mesenchyme and kidney morphogenesis. We show that *Eya1*$^{-/-}$ embryos completely lacked the blastema within the intermediate mesoderm, thus defining *Eya1* as the first gene required for the determination of metanephric blastema. In contrast, the development of mesonephros appeared to be normal in the mutant. In addition, recombinant GDNF induced UB formation from *Eya1*$^{-/-}$ Wolffian duct, indicating that the mutant Wolffian duct is functionally competent for UB outgrowth. Using a combination of both null and hypomorphic Eya1 mutants, we now demonstrate that approximately 20% of normal Eya1 protein expression is sufficient for the formation of the blastema and UB outgrowth but not for its branching. Furthermore, we show that Eya1 acts as a key regulator for *Gdnf* expression during metanephric induction and it interacts with Six1 and Pax2 to regulate the ureteric outgrowth and branching. These analyses indicate that Eya1 acts as a key regulator specifically for the determination of metanephric blastema and normal UB growth by modulating the level of *Gdnf* expression.

Materials and methods

Animals and genotyping

Eya1;Six1;Pax2 compound heterozygous mice were generated by crossing mice carrying mutant alleles of *Eya1*, *Six1* and *Pax2* and genotyping of mice and embryos was performed as previously described (Torres et al., 1995; Pichel et al., 1996; Johnson et al., 1999; Xu et al., 1999, 2003).

Phenotype analyses, in situ hybridization and antibody staining

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).

Whole-mount in situ hybridization was performed and whole-mount rudiments were sectioned by vibratome at 100 μm as described (Xu et al., 1997a).

Whole-mount immunostaining using a monoclonal antibody raised against Lim1 plus Lim2 proteins (Developmental Studies Hybridoma Bank) was performed as described (Muroyama et al., 2002). This antibody reacts with both Lim1 and Lim2 proteins (Muroyama et al., 2002).

Bead implantation and organ culture

E10.5 embryos were collected in PBS and the posterior metanephric region was dissected out. Affi-Gel blue agarose beads (100–200 mesh, 75–150 μm diameter, Bio-Rad) were incubated with 10 ng/μl of recombinant human GDNF protein (R&D) on ice for 1 h. Control beads were soaked with similar concentrations of BSA under the same conditions. Freshly isolated posterior metanephric rudiments were placed on Nucleopore filters (pore size, 0.1 mm), and protein-soaked beads were washed in PBS and placed on top of the rudiments near the mesenchymes. All explants were cultured on the filters, supported by metal grids in Dulbecco's minimal essential medium with 10% FCS at 37°C for 30 to 36 h in CO_2 incubator. After culture, the explants were fixed and processed for whole-mount in situ hybridization.

Results

In earlier work, we described the expression of *Eya1* in the kidney mesenchyme and the renal agenesis in mice lacking Eya1 (Xu et al., 1997a, 1999). Here, we present a more detailed analysis of the morphological, developmental and genetic consequences for kidney development that proceeds without the participation of normal Eya1 protein.

Eya1 specifies the metanephric blastema from caudal nephrogenic mesodermal cells

In normal mouse embryos, the metanephric blastema appears morphologically apparent as an aggregate of cells within the intermediate mesoderm at the caudal end of nephrogenic cord at E10.5 (Figs. 1A,B). However, this structure, distinct from surrounding mesenchyme, was absent in *Eya1*$^{-/-}$ embryos (Figs. 1F,G). At E11.5, the UB invades the blastema (Fig. 1C) and subsequent reciprocal interaction between these two tissues leads to the formation of a mature kidney. Similar to E10.5, all 6 E11.5 *Eya1*$^{-/-}$ embryos lacked the blastema (Fig. 1H). In contrast, the development of mesonephros appeared to be normal in the mutant by both histological and marker analyses (Figs. 1D,E,I,J). Thus, *Eya1* becomes the first defined gene necessary for the specification of the metanephric blastema.

To better understand the developmental function of Eya1 in kidney patterning, we investigated its expression pattern in normal nephrogenic mesoderm from early stages by whole-mount in situ hybridization, which has not been previously documented in detail. *Eya1* transcripts were first observed at around E8.5 in the intermediate mesoderm (Fig. 2A), which is caudal to the pro- and mesonephric anlage that express Lim1 at this stage as determined by double labeling with an antibody against Lim1/2 (Tsang et al., 2000) (Figs. 2B,C). The *Lim1* gene is initially expressed throughout the lateral plate mesoderm, intermediate mesoderm, and genital ridge from E8.0 (Bouchard et al., 2002). Within the intermediate mesoderm, *Lim1* is initially expressed in the pronephric anlage and subsequently, it becomes restricted to the Wolffian duct and mesonephric tubules (Barnes et al., 1994; Fujii et al., 1994). The *Eya1*-expressing domain within the intermediate mesoderm extends caudally along the Wolffian duct laterally, which expresses Lim1 (Figs. 2D–G). Between E9.75 and 10.5, its expression became progressively restricted to the caudal region where the UB forms (Figs. 2H,I) and by E11.5, it is strongly expressed in the induced mesenchyme around the UB (Fig. 2J). The spatiotemporal expression pattern of *Eya1* suggests that it may play a specific role in the development of the nephrogenic cord by either specifying the metanephric cell fate, determining the metanephric blastema, or regulating the metanephric induction.

To distinguish among these possibilities and determine the onset of defect in the development of mutant nephrogenic cord mesoderm, we first analyzed the expression of several mesenchyme-specific markers at E10.5 at which the blastema becomes morphologically evident. However, we failed to detect any marker expression in the mutant mesenchyme at E10.5 (Xu et al., 1999, 2003 and data not shown). The lack of metanephric mesenchyme-specific gene expression and blastema in *Eya1*$^{-/-}$ embryos suggests that Eya1 may determine the metanephric fate from nephrogenic meso-

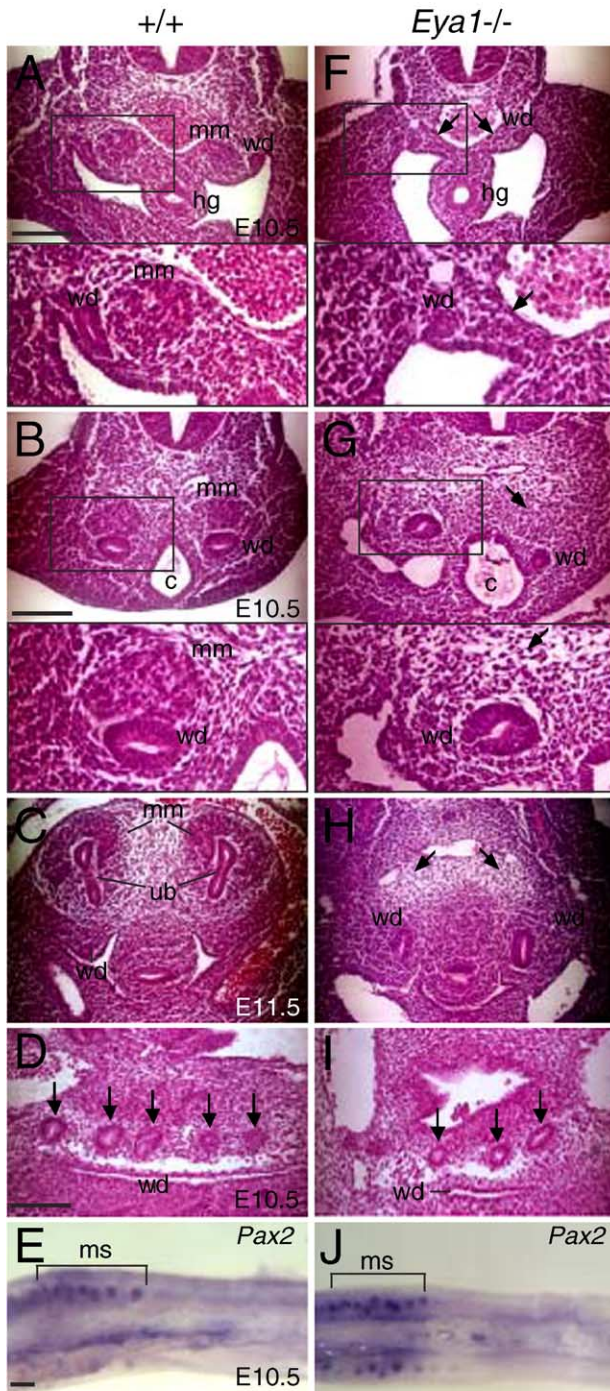

Fig. 1. *Eya1*$^{-/-}$ embryos lack the metanephric mesenchyme. (A–C) H&E stained sections from (A) anterior and (B) posterior metanephric regions of E10.5 normal embryos showing the metanephric mesenchyme or blastema (mm), and ureteric (UB) budding into the mesenchyme at E11.5 (C). Panels below A and B are higher magnification of the boxed areas. (D) An H&E stained section from E10.5 wild-type embryos showing mesonephric tubules (arrows) and (E) ventral view of a whole-mount E10.5 embryo stained with *Pax2* probe showing strong *Pax2* expression in mesonephric tubules (ms). (F, G) In E10.5 *Eya1*$^{-/-}$ embryos, no blastema (arrows) was observed whereas the Wolffian duct (wd) is apparently present. Lower panels are higher magnification of the boxed areas. (H) No UB outgrowth was observed in E11.5 *Eya1*$^{-/-}$ embryos (arrows). (I) However, mesonephric tubules (arrows) are present in the mutant and strongly express *Pax2* (J). Scale bars: 100 μm.

derm. To further examine this, we analyzed *Eya1* expression in the mutant embryos. *Eya1* mutant mice carry a targeted mutant allele that contained a neo cassette replacing the carboxy-terminal of the evolutionarily conserved Eya domain region, which contains an intrinsic protein phosphatase activity and works as a phosphatase as well as for protein–protein interaction (Xu et al., 1999; Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). *Eya1* heterozygous mice show organ defects similar to BOR syndrome, whereas the homozygous mice die at birth and completely lack ears and kidneys as well as other organs (Xu et al., 1999). RT-PCR confirmed that the transcripts containing exons downstream of the targeted deletion are not made in the homozygotes (Xu et al., 1999). Given the dominant nature of the heterozygous phenotype and no detectable normal protein in the homozygotes by Western blot (data not shown), the targeted allele is expected to eliminate wild-type Eya1 function. Using a probe specific to the 5′-end of *Eya1* cDNA, which is identical to sequences common to wild-type and *Eya1* mutant transcripts 5′ of the targeted deletion, a stable transcript is made in the homozygous embryos at E9.5–11.0 with the predicted size of a transcript containing exons (exon 1–10) upstream of the targeted deletion (Xu et al., 1999), while a normal transcript was detected in wild-type embryos but not from homozygous embryos (data not shown). Using this probe, *Eya1* transcripts were detected in the nephrogenic cord of *Eya1*$^{-/-}$ embryos at the levels similar to wild-type until E9.5 (data not shown). However, its expression is largely reduced in *Eya1*$^{-/-}$ mesoderm from E9.75 (Fig. 2K) when compared to its normal expression in the caudal nephrogenic mesoderm (arrow, Fig. 2H). At E10.5, *Eya1* expression in the metanephric region almost disappeared completely (arrows, Fig. 2L). Thus, these results indicate that the nephrogenic mesodermal cells at the caudal region are initially specified in *Eya1*$^{-/-}$ embryos, but these intermediate mesodermal cells completely fail to form the metanephric mesenchyme. Taken together, while *Eya1* appears to be dispensable for the development of pro- and mesonephroi, our data define an essential role of *Eya1* in committing mesodermal cells at the caudal end of nephrogenic cord to the metanephric fate. Failure to activate their normal differentiation program for metanephric development leads to abnormal apoptosis of the progenitor cells as detected by TUNEL assay (Xu et al., 1999).

Eya1$^{-/-}$ *Wolffian duct is uninduced for ureteric development but is functionally competent for UB formation by application of exogenous GDNF*

We have previously reported that there is no UB formation in *Eya1*$^{-/-}$ embryos (Xu et al., 1999). As the development of metanephros also depends on the proper formation of the Wolffian duct during pronephros induction, we therefore examined whether the mutant Wolffian duct is

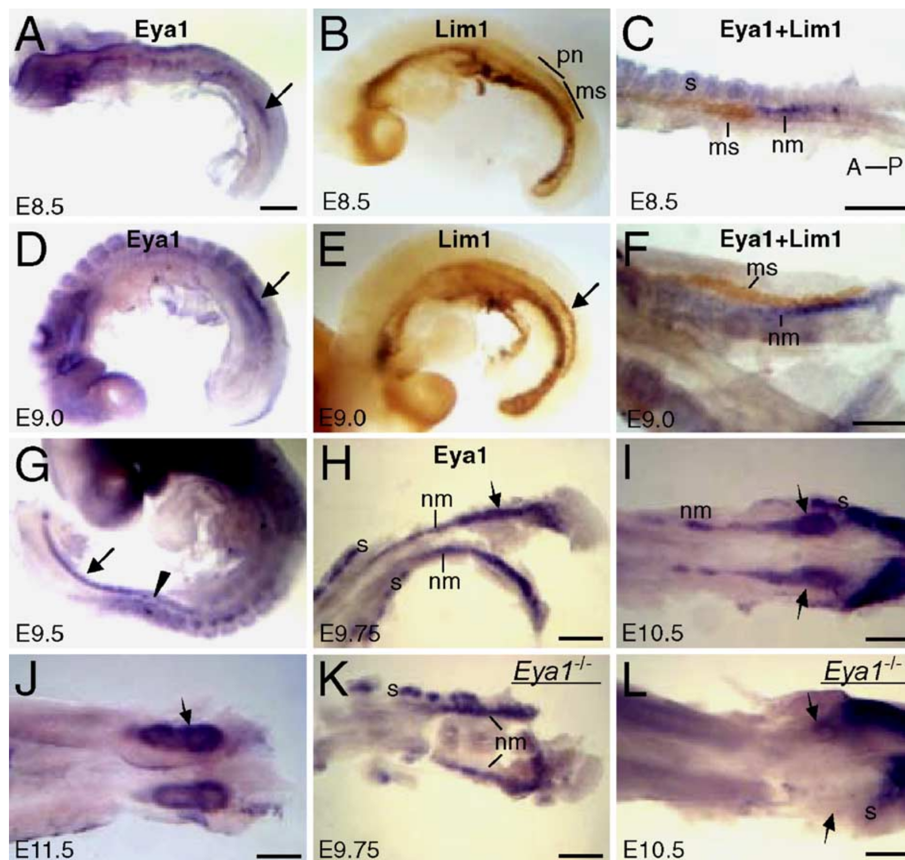

Fig. 2. The nephrogenic progenitors are specified in *Eya1*$^{-/-}$ embryos. (A) Lateral view of an E8.5 embryo showing *Eya1* staining in the presumptive nephrogenic mesenchyme (arrow) detected by whole-mount in situ hybridization. (B) Lateral view of E8.5 embryos showing Lim1 protein expression in the presumptive pro- (pn) and mesonephric (ms) anlage. This antibody also detects Lim2 protein (ventral staining in panel B). (C) Lateral view of E8.5 embryos costained with *Eya1* riboprobe and Lim1/2 antibody showing that *Eya1* expression domain in the nephrogenic mesenchyme (nm) is caudal to the Lim1 expression in the mesonephric region (ms). (D) Lateral view of E9.0 embryos showing that *Eya1* expression extends caudally in the nephrogenic mesenchyme. (E) Lateral view of E9.0 embryos showing Lim1 expression in the nephric duct including mesonephric duct (arrow). (F) Ventral view of an E9.0 embryo costained with *Eya1* probe and Lim1/2 antibody showing that *Eya1* expression in the nephrogenic mesenchyme (nm) extends caudally along the Wolffian duct laterally. Note that *Eya1* and Lim1 are not colocalized (the embryo is lightly bent due to dissection). (G) Lateral view of E9.5 embryos showing *Eya1* expression throughout the entire nephrogenic mesenchyme (arrow). Arrowhead points to the anterior limit of *Eya1* expression in the mesenchyme. (H) Ventral view of E9.75 embryos showing that *Eya1* expression becomes stronger in the caudal mesenchyme (arrow). (I) Ventral view of E10.5 embryo showing *Eya1* expression in the metanephric mesenchyme around the UB (arrow) as well as in the nephrogenic mesenchyme (nm) anterior to the kidney region and (J) by E11.5, *Eya1* expression is restricted to the metanephric mesenchyme and stronger around the UB (arrow). (K) Ventral view of E9.75 *Eya1*$^{-/-}$ embryos showing *Eya1* expression in the nephric mesenchyme (nm), but weaker than in wild-type (H). (L) However, *Eya1*-expressing cells in the nephric mesenchyme almost disappeared completely in E10.5 mutant embryos (arrows), while its expression in the somites (s) appeared normal. Scale bars: 200 μm.

functionally competent for UB outgrowth. First, we performed whole-mount in situ hybridization with epithelial markers. The UB normally emerges from caudal Wolffian duct at E10.5 and strongly expresses *c-Ret*, which is a receptor for *Gdnf* (Fig. 3A). The UB elongates further to invade the mesenchyme (Fig. 3C) and by E11.5, it undergoes branching morphogenesis to form the first T-shaped bud showing strong *c-Ret* expression (Fig. 3E). In the mutant embryos, *c-Ret* expression was observed in the Wolffian duct but the Wolffian duct is not induced for UB outgrowth (Figs. 3B,D,F). *Pax2* is normally expressed in both the mesenchyme and the ureteric epithelium (Fig. 3G) and its expression in the mutant Wolffian duct was preserved (Fig. 3H). Similarly, other epithelial markers including *Gfrα1*, *Lim1* and *Bmp7* are expressed normally in *Eya1*$^{-/-}$ Wolffian duct (data not shown). This suggests no molecular defect in *Eya1*$^{-/-}$ Wolffian duct and lack of UB induction in the mutant likely stems from the early mesenchymal defect.

Bmp4, a member of the Tgfβ superfamily of secreted signals, has been implicated in UB growth and branching by coordinating the Ret/Gfrα1/Gdnf signaling system (Dunn et al., 1997; Miyazaki et al., 2000; Raatikainen-Ahokas et al., 2000; Vainio and Lin, 2002). To exclude the possibility that misregulation of *Bmp4* expression in the mutant mesenchyme may result in defective UB formation, we analyzed its expression in *Eya1*$^{-/-}$ embryos. *Bmp4* is normally expressed in the cells surrounding the Wolffian duct and ureteric stalk (Fig. 3I) and its expression was unaffected in the mutant (Fig. 3J). Thus, the failure of UB induction in *Eya1*$^{-/-}$ embryos is *Bmp4*-independent.

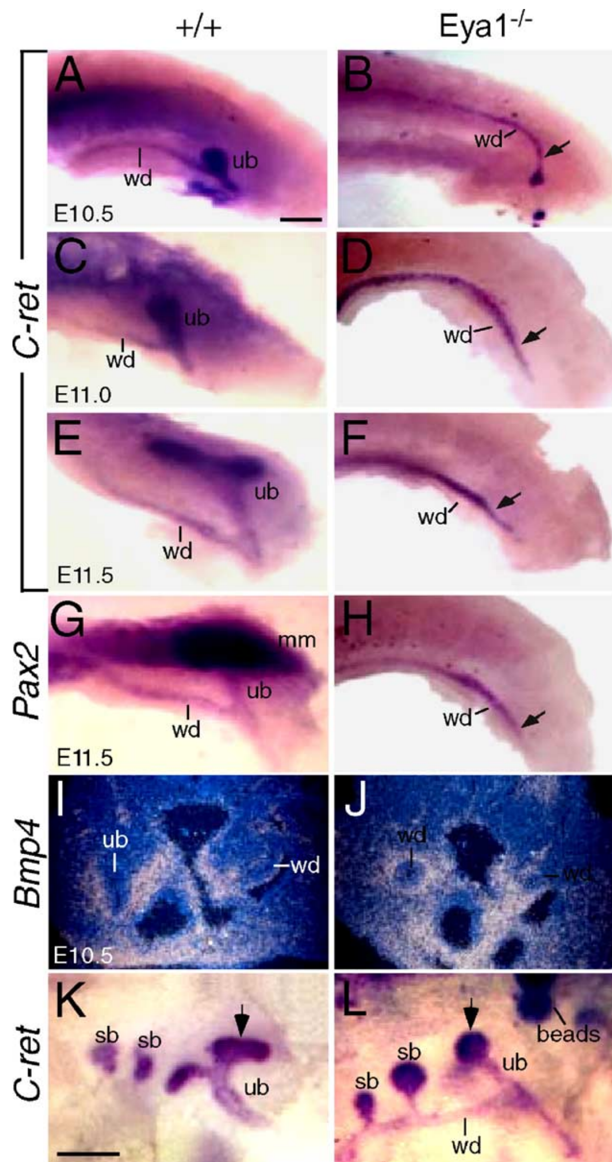

Fig. 3. $Eya1^{-/-}$ Wolffian duct is not induced for UB formation but functionally competent for UB growth by recombinant GDNF. (A–H) The metanephric region was dissected out from E10.5–11.5 embryos and stained for *c-Ret* or *Pax2* by whole-mount in situ hybridization. (A, C, E) Normal UB outgrowth at E10.5–11.0 and branching to the T-bud stage at E11.5, labeled by *c-Ret* staining. (G) E11.5 metanephric region showing strong *Pax2* expression in both the mesenchyme (mm) and ureteric epithelium. (B, D, F, H) In $Eya1^{-/-}$ embryos, the Wolffian duct (wd) expressing both *c-Ret* and *Pax2* is present but is not induced for UB formation (arrows). (I, J) Section in situ hybridization with ^{35}S-UTP showing normal *Bmp4* expression in the cells surrounding Wolffian duct (wd) and UB and its expression was unaffected in $Eya1^{-/-}$ embryos at E10.5. (K, L) Exogenous GNDF induces UB formation from $Eya1^{-/-}$ Wolffian duct. (K) A wild-type rudiment shows the branched T-shaped bud (arrow, the branched bud is slightly bent due to the positioning of the rudiment on the filter). In addition, supernumerary buds (sb) from where the Wolffian duct is not normally budding were also observed. (L) An $Eya1^{-/-}$ rudiment shows the UB outgrowth from its normal position (arrow), but failed to branch out. The tip of the bud is dilated and strongly expresses *C-ret*. Supernumerary buds (sb) were also induced in the mutant Wolffian duct. The blue dots are Affi-Gel blue agarose beads. Scale bars: 100 μm.

We next examined whether exogenous GDNF is able to induce UB outgrowth from $Eya1^{-/-}$ Wolffian duct in an organ culture system. Microdissected wild-type and E10.5 $Eya1^{-/-}$ kidney rudiments were implanted with beads containing recombinant GDNF, cultured for 30 h and then stained with *c-Ret* probe. Wild-type kidney rudiments showed the first T-bud strongly expressing *c-Ret* (arrow, Fig. 3K). Consistent with previous observations (Sainio et al., 1997; Brophy et al., 2001), a number of supernumerary buds from where the Wolffian duct is not normally budding were also observed. Interestingly, $Eya1^{-/-}$ rudiments also showed the UB outgrowth from its normal position and the formation of supernumerary buds (Fig. 3L). The UBs observed in $Eya1^{-/-}$ rudiments showed the formation of ampullae at the tip of the bud that strongly expresses *c-Ret*, but failed to branch out (Fig. 3L). No UB induction was observed when $Eya1^{-/-}$ rudiments were cultured with beads containing BSA (data not shown). This further demonstrates that $Eya1^{-/-}$ Wolffian duct is fully competent for induction by GDNF.

Eya1 acts as a critical regulator for Gdnf expression during metanephric induction

Since $Eya1^{-/-}$ embryos lack the metanephric blastema, to address whether Eya1 regulates expression levels and activities of other transcription factors or signaling molecules during metanephric induction, we used the mutant mice carrying a hypomorphic allele of *Eya1*, $Eya1^{bor}$, which is caused by the insertion of an intracisternal A particle (IAP) in intron 7 of the *Eya1* gene (Johnson et al., 1999).

In $Eya1^{bor}$ mice, heterozygotes are normal, and homozygotes exhibited kidney defects ranging from bilateral normal kidneys to unilateral absence (Johnson et al., 1999). Northern blot analysis showed a ~50% reduction in the level of normal *Eya1* transcripts in homozygotes compared with wild-type control; however, two additional transcripts were also detected in homozygotes and Eya1 protein expression has not been studied in the mutants (Johnson et al., 1999). First, to better understand the dosage effects of Eya1 protein level on the expressivity of renal phenotype, we analyzed the kidney defects in wild-type, $Eya1^{bor/+}$, $Eya1^{bor/bor}$, $Eya1^{+/-}$ carrying the knockout (null, $Eya1^{-}$) allele, $Eya1^{-/-}$ and $Eya1^{bor/-}$ carrying both alleles in C3H background. Quantitative Western blots using extracts from whole embryos at E12.5 with a specific anti-Eya1 antibody revealed that the expression levels of normal Eya1 protein in these animals are 100% in wild-type, ~80% in $Eya1^{bor/+}$ heterozygotes, ~48% in $Eya1^{+/-}$ heterozygotes, ~36% in $Eya1^{bor/bor}$ homozygotes, ~20% in $Eya1^{bor/-}$ compound heterozygotes and no expression in $Eya1^{-/-}$ homozygotes (L. Huang and P-X. Xu, in preparation). Examination of kidneys at newborn stage showed that 2 of 12 $Eya1^{+/-}$ or 1 of 14 $Eya1^{bor/+}$ heterozygous mice had slightly smaller kidneys (Table 1). Among the 7 $Eya1^{bor/bor}$ homozygous animals analyzed, severe hypoplasia was observed bilaterally in 3 animals and 2

Table 1
Kidney abnormalities in newborn compound heterozygous of *Eya1*, *Six1* and *Pax2*

Genotype	n	Duplex kidney	Small kidney	No kidney
Wild-type C3H	8	0	0	0
$Eya1^{+/-}$ ($Eya1^{ko/+}$) C3H	12	0	2[a]	0
$Eya1^{bor/+}$ C3H	14	0	1[a]	0
$Eya1^{bor/bor}$ C3H	7	0	3[b]	2 unilateral
$Eya1^{bor/ko}$ C3H	5	0	0	5 bilateral
Wild-type 129-C57BL6	27	0	0	0
$Eya1^{+/-}$ 129-C57BL6	19	0	0	3 unilateral
$Pax2^{+/-}$ 129-C57BL6	13	0	5[a]	1 unilateral
$Six1^{+/-}$ 129-C57BL6	20	0	3[c]	1 unilateral
$Eya1^{+/-};Pax2^{+/-}$ 129-C57BL6	20	2[d]	8[e]	6 unilateral, 4 bilateral
$Six1^{+/-};Pax2^{+/-}$ 129-C57BL6	21	0	5[f]	6 unilateral, 1 bilateral
$Eya1^{+/-};Six1^{+/-};Pax2^{+/-}$ 129-C57BL6	10	0	0	8 bilateral, 2 unilateral

n—Number of animals.
The kidney phenotype observed in $Eya1^{+/-};Six1^{+/-}$ double heterozygotes was similar to that seen previously (Xu et al., 2003).
[a] Number of animals showed bilaterally smaller kidneys with reduction of 10–15% in weight.
[b] Number of animals showed bilaterally smaller kidneys with reduction of 70–80% in weight (severe hypoplasia).
[c] Three $Six1^{+/-}$ animals showed unilaterally smaller kidneys with reduction of approximately 10% in weight.
[d] Two $Eya1^{+/-};Pax2^{+/-}$ double heterozygotes showed duplex kidneys with double ureters.
[e] Eight $Eya1^{+/-};Pax2^{+/-}$ double heterozygotes showed smaller kidneys either bilaterally (n = 6) or unilaterally (n = 2), with reduction of approximately 20–50% in weight.
[f] Five $Six1^{+/-};Pax2^{+/-}$ compound heterozygotes showed small kidneys either bilaterally (n = 4) or unilaterally (n = 1), with reduction of approximately 20–70% in weight.

showed unilateral agenesis (Table 1). Interestingly, 100% of $Eya1^{bor/-}$ compound heterozygotes showed bilateral agenesis (Table 1), demonstrating that ~20% of normal Eya1 protein level is below the critical threshold necessary for the development of any kidney structures.

To determine the developmental failure of kidney formation in $Eya1^{bor/bor}$ and $Eya1^{bor/-}$ compound heterozygous mice, we analyzed these mutants at earlier stages. The metanephric blastema was morphologically normal in all 4 $Eya1^{bor/bor}$ embryos at E10.5 (data not shown). At E11.5, the ureteric budding into the mesenchyme and the induction of branching morphogenesis occurred normally in 3 out of 4 $Eya1^{bor/bor}$ embryos analyzed (Figs. 4A,B) but only occurred unilaterally in one $Eya1^{bor/bor}$ embryo (data not shown). However, reduced branching was observed in 2 of 5 $Eya1^{bor/bor}$ embryos from E13.5. These observations would explain the hypoplasia and unilateral agenesis observed in newborn animals.

In $Eya1^{bor/-}$ compound mutant embryos, the metanephric blastema was formed at E10.5 in all 5 embryos analyzed (Figs. 4C,D). The UB was also formed but it failed to branch out by histological and marker analyses (n = 8, Figs. 4E–H). Interestingly, *C-ret* expression was significantly reduced at the tip of the bud (arrow, Fig. 4F), which normally expresses *C-ret* at a higher level (arrow in Fig. 4E). Thus, ~20% of normal Eya1 protein level is sufficient for the specification of the metanephric mesenchyme from intermediate mesodermal cells and for UB outgrowth but not for its branching.

We have previously reported that *Eya1* expression is normal in $Pax2^{-/-}$ or $Six1^{-/-}$ blastema and *Six1* expression is also normal in $Pax2^{-/-}$ blastema but *Pax2* expression in $Six1^{-/-}$ blastema is markedly reduced by section in situ hybridization (Xu et al., 2003). However, recent report by Li et al. (2003) showed that *Pax2* expression level was unaffected in *Six1*-null kidney mesenchyme on sections. After confirming the formation of metanephric blastema in $Eya1^{bor/-}$ mutant mice, we sought to clarify whether Eya1 regulates the expression of *Pax2* or *Six1* and whether *Six1* regulates *Pax2* expression by whole-mount in situ hybridization. *Pax2* is expressed in the entire nephrogenic mesoderm at E10.5 and strongly in the metanephric mesenchyme in wild-type embryos (Fig. 4I). In $Eya1^{bor/-}$ embryos, its expression in the nephrogenic mesoderm appears to be unaffected whereas only very weak expression was observed in the caudal region where the UB forms (arrow, Fig. 4J). In agreement with our previous observations, obvious *Pax2* expression was undetectable in the metanephric mesenchyme of all 6 $Eya1^{-/-}$ or $Six1^{-/-}$ embryos (Figs. 4K,L), but its expression in the ureteric epithelium and the nephrogenic mesoderm anterior to the metanephric region was preserved (Figs. 4K,L). *Six1* expression in $Eya1^{bor/-}$ metanephric mesenchyme was also largely reduced (Figs. 4M,N). Taken together, these results further demonstrate that *Six1* is required for normal expression of *Pax2* in the metanephric mesenchyme. Using $Eya1^{bor/-}$ compound mutants, we now demonstrate that Eya1 regulates normal expression of *Six1* and *Pax2* in the metanephric mesenchyme.

We next analyzed whether this early arrest of UB development observed in $Eya1^{bor/-}$ embryos is caused by downregulation of *Gdnf*. In $Eya1^{-/-}$ embryos, *Gdnf* expression was markedly reduced from E9.5 by both whole-mount and section in situ hybridization (Xu et al., 1999 and data not shown). Consistent with the observation of UB outgrowth in $Eya1^{bor/-}$ embryos, *Gdnf* is expressed in the mutant mesenchyme at reduced levels in all 4 embryos (arrow, Figs. 5A–D). Thus, Eya1 protein expression level critically affects *Gdnf* expression and the low amount of Gdnf made by $Eya1^{bor/-}$ mesenchyme is sufficient for inducing UB outgrowth but not for its normal branching. Alternatively, the complete failure of branching morphogenesis may be partly caused by defective expression of other mesenchymal factors that are necessary for the initiation of branching morphogenesis.

Previous studies have shown that *Gdnf* expression was absent in $Pax2^{-/-}$ blastema and Pax2 regulates *Gdnf* transcription in vitro (Brophy et al., 2001). However, our observation that *Gdnf* expression is reduced in $Six1^{-/-}$

blastema that lacks strong *Pax2* expression suggests that *Gdnf* expression in the blastema does not absolutely require Pax2 (Fig. 4; Xu et al., 2003). In agreement with this, we have found that it is expressed in the $Pax2^{-/-}$ mesenchyme at a reduced level (Figs. 5E,F). The reduced *Gdnf* expression in $Pax2^{-/-}$ blastema suggests that Pax2 probably stimulates *Gdnf* expression.

We further analyzed the expression of *Eya1*, *Six1* and *Pax2* in *Gdnf*-null embryos to clarify the genetic relationship between these genes in the mesenchyme. *Eya1* expression in normal metanephric mesenchyme at E10.5 extends substantially anterior (Fig. 5G) when compared with the restricted *Gdnf* expression (Fig. 5A) and its expression is unaffected in $Gdnf^{-/-}$ mesenchyme (n = 4, Fig. 5H). Similarly, the expression level of both *Six1* and *Pax2* appeared to be unaffected in E10.5 $Gdnf^{-/-}$ mesenchyme of all 8 embryos analyzed by both whole-mount and section in situ hybridization (Figs. 5I–L and data not shown). Collectively, our data indicate that *Eya1* acts as a critical regulator for *Six1*, *Pax2* and *Gdnf* expression and it may also act together with *Six1* or *Pax2* to maintain normal expression of *Gdnf* in the mesenchyme.

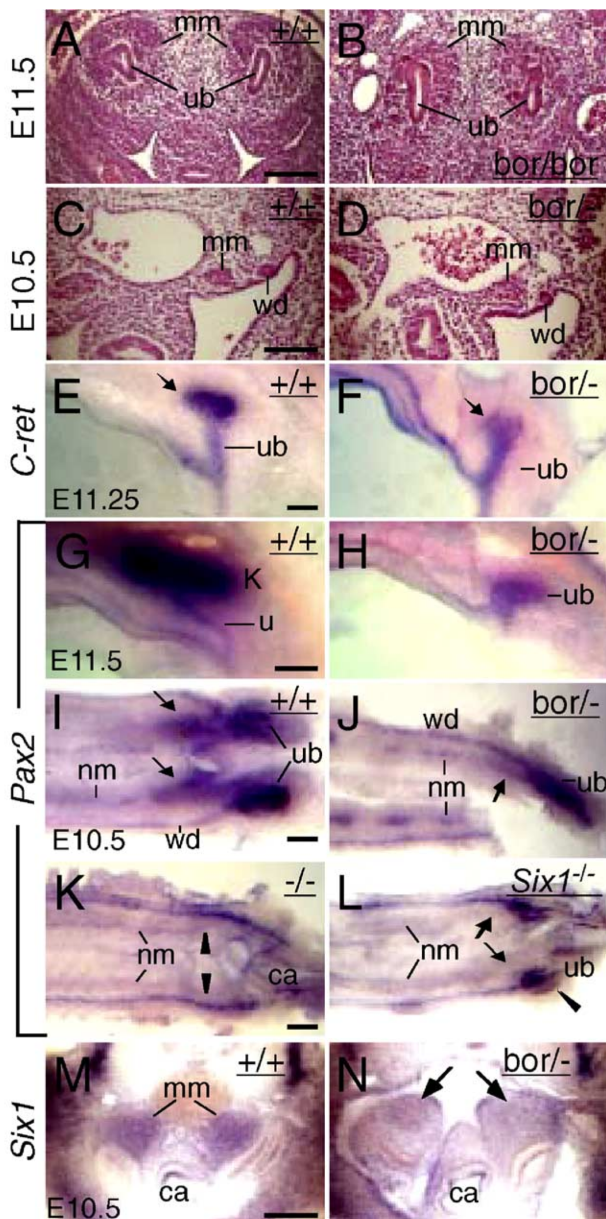

Fig. 4. *Eya1* regulates *Gdnf*, *Six1* and *Pax2* expression during UB outgrowth. (A–D) Histological sections from metanephric regions at E10.5–11.5. (A) Normal UB budding into the mesenchyme (mm) at E11.5. (B) An E11.5 $Eya1^{bor/bor}$ embryo showing UB branching on both sides. (C) Normal formation of the metanephric blastema (mm) at E10.5. (D) In $Eya1^{bor/-}$ embryos, the blastema (mm) is specified. (E–L) Whole-mount embryos. Lateral view for panels E–H, and ventral view for panels I–L. Anterior is to the left. (E) Normal UB invasion into the mesenchyme and branching morphogenesis has been induced at E11.25, as labeled by *C-ret* staining. (F) In $Eya1^{bor/-}$ embryos, UB outgrows normally but its branching morphogenesis is disrupted and *C-ret* expression becomes weaker at the tip of the bud (arrow). (G) *Pax2* is expressed in the UB and mesenchyme in E11.5 wild-type embryos (G). (H) In $Eya1^{bor/-}$ embryos, its expression in the UB is normal but no obvious expression is seen in the mesenchyme. (I) *Pax2* is strongly expressed in the caudal metanephric mesenchyme (arrows) as well as in the anterior nephric mesenchyme (nm) and Wolffian duct (wd) in E10.5 wild-type embryos. (J–L) In $Eya1^{bor/-}$ (J), $Eya1^{-/-}$ (K) or $Six1^{-/-}$ (L) embryos, *Pax2* expression in the metanephric mesenchyme is markedly reduced (arrows in J, L) or undetectable (K), but its expression in the anterior mesenchyme (nm) and Wolffian duct (wd) is relatively normal. Arrowheads in panel K point to the caudal limit of *Pax2* expression in the mesenchyme. Arrowhead in panel L points to the UB formation in the *Six1* mutants. (M, N) Sections of whole-mount embryos showing that *Six1* expression in the metanephric mesenchyme (mm, M) is also reduced in $Eya1^{bor/-}$ mutants (arrows). Note that *Six1* is widely expressed in the urogenital ridge region. ca, cloaca. Scale bars: 100 μm.

Eya1, Six1 and Pax2 function in a molecular pathway to regulate Gdnf expression during UB growth and branching

To further test whether *Eya1*, *Six1* and *Pax2* function in a molecular network to mediate the competence of the mesenchyme for UB growth and branching by regulating *Gdnf* expression, we examined the kidneys of newborn compound heterozygotes of *Eya1;Pax2*, *Six1;Pax2* and *Eya1;Six1;Pax2* (Table 1). Double heterozygous animals showed variable renal defects including hypoplasia, duplex kidneys and double ureters, and agenesis (Figs. 6A–D). In contrast to the mild renal abnormalities observed in each single heterozygous animals, 10 of 20 (50%) $Eya1^{+/-};Pax2^{+/-}$ and 7 of 21 (33%) $Six1^{+/-};Pax2^{+/-}$ double heterozygous animals showed renal agenesis (Table 1). Renal agenesis was either unilateral or bilateral. In some $Six1^{+/-};Pax2^{+/-}$ animals associated with renal agenesis, ureters that end blindly were observed (arrows, Fig. 6D), similar to that seen in some $Eya1^{+/-};Six1^{+/-}$ animals (Xu et al., 2003). Interestingly, 10% of $Eya1^{+/-};Pax2^{+/-}$ mice showed duplex kidneys and double ureters (Fig. 6B). This phenotype most likely results from a misregulation of *Gdnf* expression by *Eya1* and *Pax2*, as altered expression of *Gdnf* has been shown to induce additional ureters (Kume et al., 2000; Grieshammer et al., 2004). Histological

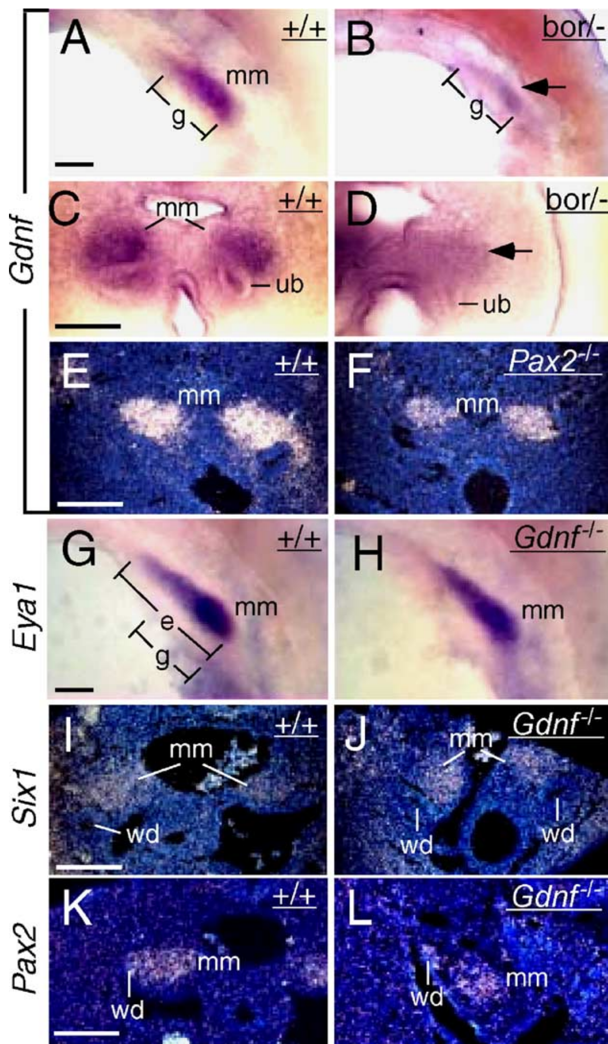

Fig. 5. *Eya1* controls *Gdnf* expression in the mesenchyme. (A, B) Lateral views of whole-mount embryos showing restricted *Gdnf* expression in the metanephric mesenchyme (mm) in E10.5 wild-type embryos and (B) its expression is reduced in $Eya1^{bor/-}$ embryos (arrow). (C, D) Sections of whole-mount embryos showing *Gdnf* expression in the normal mesenchyme (mm) and its weak expression in $Eya1^{bor/-}$ mesenchyme (arrow). (E, F) Section in situ showing *Gdnf* expression in the mesenchyme in wild-type embryos (E, F) its reduced expression in $Pax2^{-/-}$ mesenchyme. (G, H) Lateral views of whole-mount embryos showing similar *Eya1* expression in the mesenchyme (mm) between wild-type (G) and all 4 $Gdnf^{-/-}$ embryos (H). Note that *Eya1* expression domain extends more anteriorly (e) than restricted domain of *Gdnf* expression (g, also in panels A, B). (I, J) *Six1* expression in the mesenchyme is similar between wild-type (I) and *Gdnf* mutant embryos (J). (K, L) *Pax2* expression in mesenchyme is also similar between wild-type (K) and *Gdnf* mutant (L) embryos at E10.5. Scale bars: 100 μm.

analysis of $Eya1^{+/-};Pax2^{+/-}$ or $Six1^{+/-};Pax2^{+/-}$ newborn hypoplastic kidneys confirmed that there are fewer nephrons but all developing structures are present (data not shown), suggesting that the kidney hypoplasia observed in the double heterozygotes may result from a reduced induction of branching morphogenesis of the UB. In contrast to the variable renal phenotype observed in the double heterozygotes, 100% of $Eya1^{+/-};Six1^{+/-};Pax2^{+/-}$ triple heterozygotes showed renal agenesis (Table 1). Among the 10 triple heterozygous animals analyzed, only 2 showed slightly reduced branching unilaterally. Together, these data strongly suggest that *Eya1*, *Six1* and *Pax2* genetically interact and function in a molecular pathway during kidney development.

To examine the basis of renal agenesis associated with $Eya1^{+/-};Six1^{+/-};Pax2^{+/-}$ heterozygotes, we analyzed the mutant kidney development at early stages. Histological analysis showed that the metanephric mesenchyme was well formed (Fig. 6F). The UB formation occurred normally and it invaded or contacted the mesenchyme but no branching morphogenesis occurred in all 3 embryos analyzed (Figs. 6E,F), similar to that seen in $Eya1^{bor/-}$ mutant embryos. Whole-mount in situ hybridization with *C-ret* probe further confirmed outgrowth of the UB in all 4 triple heterozygous embryos at E10.5 (Figs. 6G,H), but it failed to branch out at E11.5 as labeled with *C-ret* ($n = 3$, Fig. 6J) and *Pax2* ($n = 4$, Fig. 6L). This early defective branching morphogenesis suggests a deficiency in the transduction of a signal derived from the mesenchyme.

Recent studies have shown that a combination of Gdnf and other unknown mesenchymal factors is necessary for triggering branching morphogenesis (Qiao et al., 1999), we therefore sought to examine whether the failure of UB branching observed in the triple mutants is caused by downregulation of *Gdnf* expression. Consistent with the observation of UB outgrowth, *Gdnf* expression was detected in the triple heterozygous embryos from E10.5 ($n = 4$ for each stage) but its expression level appeared to be reduced (Figs. 6N,P). Quantitative RT-PCR revealed approximately 45% reduction in *Gdnf* expression level in E10.5 triple heterozygous mesenchyme compared to wild-type mesenchyme (data not shown). These results demonstrate that *Eya1*, *Six1* and *Pax2* genetically interact and regulate the mesenchymal production of Gdnf during UB growth and branching.

Discussion

Despite a large number of genes have been identified as regulators of kidney organogenesis, targeted mutagenesis has failed to demonstrate an essential role for any of these genes in controlling the specification of the metanephric mesenchyme, the earliest step of metanephric kidney development. Here, we demonstrated that Eya1 is the key regulator for the specification of the blastema and the expression of *Gdnf*. Moreover, we show that *Eya1*, *Six1* and *Pax2* function in a molecular pathway to mediate the competence of the mesenchyme for normal UB branching by modulating *Gdnf* expression.

The role of Eya1 in the specification of metanephric blastema

All kidney development requires proper specification of nephrogenic cord mesoderm. The Wolffian duct differ-

entiates from the nephrogenic cord and it induces pro- and mesonephric tubules as it extends caudally towards the cloaca and produces a UB during metanephric development. Recent studies have found that Lim1 and Pax2/Pax8 are involved in the specification of the nephrogenic mesoderm. It has been suggested that the Lim1 protein probably acts as a competence factor to determine the nephric field, within which the local activation of Pax2 and Pax8 specifies the kidney fate (Tsang et al., 2000; Bouchard et al., 2002). Unlike Lim1 or Pax2/Pax8, Eya1 appears to be dispensable for the pro- and mesonephric development and the formation of Wolffian duct. Our analyses revealed a genetic requirement for *Eya1* in the creation of the metanephric blastema, the earliest stage of metanephric kidney development. Therefore, *Eya1* becomes the first defined gene necessary for the specification of the metanephric fate within the nephrogenic field.

Formation of the metanephric blastema is the first morphological sign of metanephric differentiation within the intermediate mesoderm. To date, however, little is known about how exactly the blastema is specified and when the metanephric cell fate is determined. As Gdnf acts as a mesenchyme-derived ligand through its receptors Ret and Gfrα1 expressed in the Wolffian duct to induce UB outgrowth, several models which restrict *Gdnf* expression domain for localizing UB formation to the appropriate site have been recently proposed. However, it is unclear how *Gdnf* expression is activated. In this study, we carefully assessed the onset of *Eya1* expression during the development of nephrogenic mesoderm, its relation with *Gdnf* expression and the presence of nephrogenic mesodermal cells in $Eya1^{-/-}$ embryos. Our data show that *Eya1* is expressed in the intermediate mesoderm next to the presumptive pro- and mesonephric anlage from as early as E8.5, and becomes progressively restricted to the metanephric mesenchyme. This spatiotemporal expression pattern is strikingly similar to that of *Gdnf* (Pichel et al., 1996; Sanchez et al., 1996; Grieshammer et al., 2004). Furthermore, *Gdnf* expression appears to be *Eya1* dosage dependent, whereas *Eya1* expression is independent from *Gdnf* (Fig. 5; Xu et al., 1999), therefore, *Eya1* is genetically upstream of *Gdnf* and acts as a positive regulator for its activation. Consistent with this, while both genes appear to be only necessary for the metanephric development (Pichel et al., 1996; Sanchez et al., 1996), the defect occurs earlier

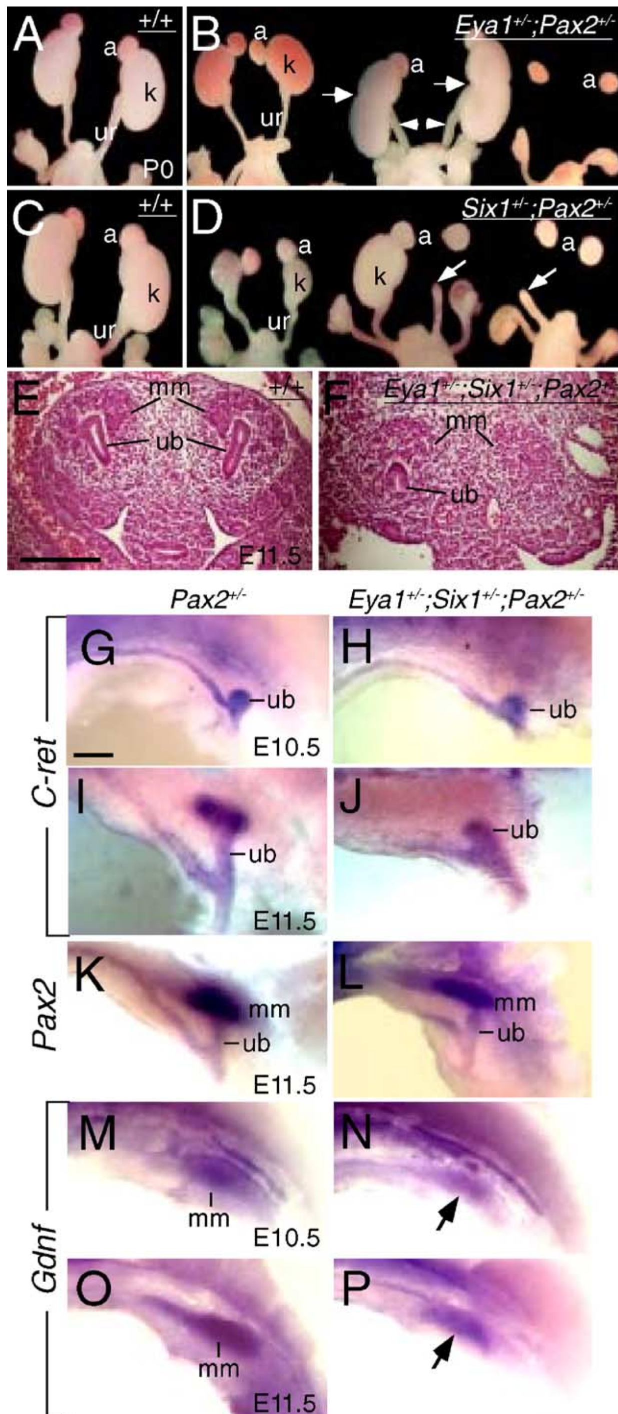

Fig. 6. *Pax2* genetically interacts with *Eya1* and *Six1* during kidney organogenesis. (A–D) P0 wild-type (A, C), $Eya1^{+/-}$;$Pax2^{+/-}$ (B) and $Six1^{+/-}$;$Pax2^{+/-}$ (D) kidneys (k). $Eya1^{+/-}$;$Pax2^{+/-}$ or $Six1^{+/-}$;$Pax2^{+/-}$ animals showed either unilateral or bilateral renal hypoplasia or agenesis. 10% of $Eya1^{+/-}$;$Pax2^{+/-}$ animals showed duplex kidneys (arrows, B) with double ureters (arrowheads, B). In some $Six1^{+/-}$;$Pax2^{+/-}$ animals associated with renal agenesis, ureters that end blindly were observed (arrows, D). Adrenal glands (a) and genital tracts appeared normal in all compound heterozygous animals analyzed. (E) Histological section showing normal UB invasion into the mesenchyme and branching at E11.5. (F) A section of *Eya1;Six1;Pax2* triple heterozygous embryos showing partial UB invasion. No branching morphogenesis occurred in these mutants. (G–P) Whole-mount in situ hybridization. (G–J) *C-ret* staining showing UB formation at E10.5 (G) and its branching at E11.5 (I) in $Pax2^{+/-}$ embryos. In the triple heterozygotes, UB formation is normal at E10.5 (H) but it failed to branch out at E11.5 (J). (K, L) *Pax2* staining showing that UB invade into the mesenchyme in $Pax2^{+/-}$ embryos (K), but in the triple heterozygotes, the UB showed a decreased growth and failed to invade the mesenchyme completely (L). (M–P) *Gdnf* is expressed in the mesenchyme at E10.5 (M) and its expression level is increased at E11.5 in $Pax2^{+/-}$ embryos (O). In the triple heterozygotes, *Gdnf* is expressed in the mesenchyme but its expression level is decreased from E10.5 (arrow, N, P). Scale bars: 100 μm.

and more severe in the *Eya1* mutants than in the *Gdnf* mutants. Our observation of a complete absence of the blastema in *Eya1*$^{-/-}$ embryos suggests that Eya1 may be involved in the initial selection of metanephric cell fate within the intermediate mesoderm. When Eya1 is activated in the nephric field, which is specified by Lim1, it will activate *Gdnf* expression and trigger metanephric cell fate program. Therefore, the metanephric cell fates are probably assigned in the intermediate mesoderm from as early as E8.5. As the Wolffian duct induces nephric differentiation, the pro-, meso- and metanephric cell fates are probably induced sequentially along the caudal extension of the Wolffian duct. Here, we demonstrated that the nephric progenitors are specified in *Eya1*$^{-/-}$ embryos as labeled with *Eya1* expression. The presence of mesenchymal progenitors in the mutant suggests that Eya1 is not required for determining metanephric cell fate and it probably acts in combination with other factors, such as *Lim1*, *Pax2* or *Pax8*, to induce *Gdnf* expression at this early stage.

How a group of progenitor cells at the caudal end of the nephrogenic cord aggregate to from the blastema, which appears almost 2 days after *Eya1* and *Gdnf* expression is activated, and how Eya1 acts to regulate its formation? Since recent genetic studies suggest that the restriction of *Gdnf* expression domain in the blastema is probably achieved by downregulation of its expression in the anterior region (Kume et al., 2000; Grieshammer et al., 2004), a likely potential model by which Eya1 acts to mediate metanephric differentiation is that the progenitors proliferate along the A–P axis and a subpopulation of the cells at the caudal end condense to form the blastema when *Gdnf* expression becomes restricted to those cells. Consistent with this model, we have found that *Pax2* expression in *Eya1*$^{bor/-}$ mutants was normal in the anterior mesenchyme but was reduced in the metanephric mesenchyme (Fig. 4). Eya1 may regulate cell aggregation and failure to activate their normal differentiation program, the precursors are probably eliminated by cell death, as detected by TUNEL assay (Xu et al., 1999).

It should be noted that *Eya1* is broadly expressed in the nephrogenic mesenchyme. However, only a subpopulation of these cells at the caudal end will take a metanephric differentiation fate and maintain *Eya1* expression. The expression of *Eya1* does not therefore suffice to generate metanephric mesenchyme, and additional signals are required. One possible explanation is that the function of Eya1 might be modified in cells that will form metanephric blastema, for instance by a phosphorylation event controlled by signals that induce metanephric differentiation or aggregation. Alternatively, Eya1 might cooperate with additional factors regulated by such signals. Recently, several genes that are involved in restricting *Gdnf* expression to the metanephric blastema along the A–P axis have been reported (Kume et al., 2000; Grieshammer et al., 2004); however, how these signals are received and transmitted by the intermediate mesoderm remains obscure. Eya1 may work together with other factors in transmitting the specific positioning information into metanephric specification. For instance, the broad *Pax2* expression in the anterior nephric mesenchyme may be involved in restricting *Gdnf* expression, as discussed below.

In summary, our finding that Eya1 is essential for differentiation of metanephric cell fate suggests that Eya1 protein may control the gene expression program responsible for the metanephric differentiation. Consistent with this view, mutations in all other mesenchymal genes do not appear to affect the differentiation of mesodermal cells into blastema (Kreidberg et al., 1993; Dudley et al., 1995; Pichel et al., 1996; Sanchez et al., 1996; Dudley and Robertson, 1997; Dudley et al., 1999; Nishinakamura et al., 2001; Brophy et al., 2001; Wellik et al., 2002; Esquela and Lee, 2003; Xu et al., 2003). It will be important to gain molecular insight into the regulation of *Eya1* by identifying its essential kidney-specific enhancers and upstream regulatory factors, using transgenic approaches.

Eya1, Six1, Pax2 and Gdnf/Ret signals in establishing the competence of the blastema for UB outgrowth and branching morphogenesis

Our data demonstrate that the formation of a functional metanephric blastema depends on Eya1, and moreover, is even dosage dependent. In the absence of the blastema, the induction of caudal Wolffian duct for UB development is not initiated. Interestingly, results obtained from *Eya1*$^{bor/-}$ mice demonstrate that ~20% of normal Eya1 protein expression level is sufficient for specifying the blastema and the UB outgrowth but not for its normal branching.

As we have found that the blastema forms in *Eya1*$^{bor/-}$ mice, this allowed us to assess the regulatory relation with *Six1*, *Pax2* and *Gdnf*. Our data show that *Eya1* appears to regulate the expression of *Six1*, *Pax2* and *Gdnf*. Analysis of null mutations in *Six1* by two groups resulted in controversy over whether *Pax2* is expressed normally in the metanephric mesenchyme, which is formed in the mutants. These analyses were done on section in situ hybridization. As *Pax2* is expressed in the entire nephric mesenchyme and the Wolffian duct, it may be difficult to distinguish between the metanephric mesenchyme and the anterior nephric mesenchyme on sections. Using whole-mount in situ hybridization, we have found that *Pax2* expression is reduced specifically in the mesenchyme where the UB forms, whereas its expression is preserved in the nephric mesenchyme anterior to the metanephric region. Thus, this result, combined with our previous data (Xu et al., 2003), conclusively demonstrates that *Six1* is required for normal expression of *Pax2* in the metanephric mesenchyme but not in the anterior nephric mesenchyme. Therefore, while *Pax2* has an early role in the pro- and mesonephric development, it appears to function downstream of the *Eya1–Six* regulatory pathway in the metanephric patterning.

The branching defects observed in *Eya1*$^{bor/-}$ mice are associated with a reduction in mesenchymal *Gdnf* expression. Given that Gdnf can function as a chemoattractant, low Gdnf levels may result in lower outgrowth promoting activity as observed in *Eya1*$^{bor/-}$ mutants. Failure to undergo normal branching morphogenesis, the mutant ureteric tips subsequently lose *C-ret* expression, which is normally expressed robustly in that site during all stages of metanephric development (Fig. 4). Thus, ~20% of normal Eya1 expression level is below the critical threshold for maintaining Ret/Gdnf signal levels to support branching morphogenesis. Recently, *Gdf11* and *Hox11* genes have been shown to regulate the expression of *Gdnf* but not *Eya1* during UB outgrowth and branching (Wellik et al., 2002; Esquela and Lee, 2003). Thus, Eya1 probably controls the gene expression program responsible for establishing the competence of the blastema and interacts with other regulators to control *Gdnf* expression for normal UB outgrowth and branching. Our data presented above together with previous observations (Brophy et al., 2001) indicate that Pax2 may directly regulate *Gdnf* expression during branching morphogenesis. Although the mechanistic details of how do these factors regulate *Gdnf* expression at this critical stage of kidney development are unclear, it is possible that they may form a multimeric complex to regulate the optimum level of *Gdnf* expression. An alteration in the expression level of any of these molecules may have a direct effect on *Gdnf* expression in vivo. The observations of duplex kidneys with double ureters in some *Eya1;Pax2* double heterozygous animals, reduced branching morphogenesis in the hypoplastic kidneys of *Eya1;Pax2* or *Six1;Pax2* and renal agenesis in all *Eya1;Six1;Pax2* triple heterozygous animals further support this notion.

By quantitative RT-PCR, we found that *Gdnf* expression was reduced to ~45% in the triple mutant mesenchyme (data not shown). As *Gdnf* heterozygous animals exhibited a wide range of renal defects, including unilateral hypoplasia, unilateral agenesis and severe bilateral dysgenesis (Pichel et al., 1996), the branching morphogenesis defect observed in the triple mutants that express ~55% of normal *Gdnf* mRNA level suggests that additional factors necessary for normal branching morphogenesis are affected in the mutants. The mesenchymal factors Pleitrophin (Ptn) and Integrin α8 have been shown to be essential for ureteric growth and branching (Müller et al., 1997; Patterson et al., 2001; Sakurai et al., 2001). Further expression studies of *Ptn*, *Integrin α8*, *Gdf11* and *Hox11* genes in the triple heterozygotes as well as in *Eya1*$^{bor/-}$ mutants that show renal agenesis (100%), will clarify whether they are also under the control of these transcriptional factors.

It should be noted that 10% of *Eya1;Pax2* double heterozygous mutants showed duplex kidneys and double ureters and the ureters insert properly into the bladder, very similar to that seen in the *Foxc1* mutants (Kume et al., 2000). This phenotype is likely caused by abnormal maintenance of *Gdnf* expression in the region anterior to the site of normal UB formation. This observation raises the possibility that Pax2 may be required to repress *Gdnf* expression in the anterior region. In support of this, we have found that *Pax2* expression is normal in *Eya1*$^{bor/-}$ and *Six1*$^{-/-}$ nephric mesenchyme anterior to the metanephric region and no supernumerary UBs or double ureters were observed in these mutants. As *Foxc1* expression also appeared to be normal in these mutants (data not shown), it is possible that Pax2 and Foxc1 act together to regulate the restriction of *Gdnf* expression to the blastema.

In summary, our analyses revealed the dosage effects of Eya1 protein level on the expression of the morphogen *Gdnf*, whose threshold critically affects the expressivity of kidney phenotype. These results provide molecular and developmental bases for explaining the clinical severity of human BOR diseases.

Acknowledgments

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