COUP-TFII is essential for metanephric mesenchyme formation and kidney precursor cell survival

Cheng-Tai Yu¹, Ke Tang¹, Jae Mi Suh¹*, Rulang Jiang³, Sophia Y. Tsai¹,²,⁺ and Ming-Jer Tsai¹,²,⁺

SUMMARY
Development of the metanephric kidney in mammals requires complex reciprocal tissue interactions between the ureteric epithelium and the mesenchyme. It is believed that Gdnf, produced in the metanephric mesenchyme, activates Ret signaling in the Wolffian duct to initiate the formation of the metanephros. However, the molecular mechanism for induction of Gdnf in the metanephric mesenchyme is not completely defined. Previous studies demonstrated that during the early stages of kidney development, loss of Osr1, Eya1, Pax2 or Wt1 gene function in the metanephric mesenchyme compromises the formation of the kidney. Moreover, it has been shown that the Hox11-Eya1-Pax2 complex activates the expression of Six2 and Gdnf in the metanephric mesenchyme to drive nephrogenesis. Here, we demonstrate that the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII, also known as Nr2f2) is required for the specification of the metanephric mesenchyme. Deletion of COUP-TFII at E7.5 results in improper differentiation of the metanephric mesenchyme and absence of essential developmental regulators, such as Eya1, Six2, Pax2 and Gdnf. Importantly, we show that COUP-TFII directly regulates the expression of both Eya1 and Wt1 in the metanephric mesenchyme. Our findings reveal, for the first time, that COUP-TFII plays a central role in the specification of metanephric fate and in the maintenance of metanephric mesenchyme proliferation and survival by acting as a crucial regulator of Eya1 and Wt1 expression.

KEY WORDS: COUP-TFII, Metanephric mesenchyme, Eya1, Wt1, Mouse

INTRODUCTION
The mammalian kidney originates from the intermediate mesoderm and develops through three distinct stages: pronephros, mesonephros and metanephros. Organogenesis of the permanent kidney occurs through reciprocal interactions between the Wolffian (mesonephric) duct and the metanephric mesenchyme. At embryonic day (E) 10.5 in mouse, the metanephric mesenchyme secretes glial cell line-derived neurotrophic factor (Gdnf), which induces the Wolffian duct to invade the metanephric mesenchyme and induce adjacent mesenchymal cells to condense. These condensed metanephric mesenchyme cells then induce ureteric bud growth and branching, leading to the formation of nephrons (Dressler, 2006; Saxén, 1987). Specifically, Gdnf promotes ureteric bud growth by binding to its receptor tyrosine kinase (Ret) and co-receptor Gdnf family receptor alpha-1 (Gfrα1) on the surface of the ureteric bud (Durbec et al., 1996; Moore et al., 1996; Sainio et al., 1997; Vainio and Lin, 2002).

Although it is well established that the Gdnf-Ret signal transduction pathway initiates metanephric induction, the detailed mechanism of this signal pathway is still not well understood. During the metanephric mesenchyme condensation, many marker genes, including Osr1 (James et al., 2006), Pax2 (Torres et al., 1995), Eya1 (Sajithlal et al., 2005), Wt1 (Kreidberg et al., 1993), Six1 (Xu et al., 2003), Six2 (Self et al., 2006) and the Hox11 paralogous group (Hoxa11, Hoxc11 and Hoxd11) (Wellik et al., 2002), are expressed in the metanephric mesenchyme. Ablation of any of these genes in mice leads to renal agenesis, indicating that these genes are essential for the proper formation of the kidney. Osr1 and Eya1, the two earliest genes expressed in kidney precursor cells, sit atop the signal cascade and activate expression of other marker genes. Osr1 is expressed first. Mice lacking Osr1 do not form metanephric mesenchyme and do not express Eya1, Six2, Pax2, Sall1 and Gdnf. Eya1 is expressed downstream of Osr1 and is required for renal genesis (Xu et al., 1999). Eya proteins have no intrinsic DNA-binding domain but can localize to the nucleus and function as transcriptional co-activators (Ohto et al., 1999). Eya1 not only works with Six1 and Pax2 to regulate Gdnf expression, but also complexes with Hox11 paralogous proteins (including Hoxa11, Hoxc11 and Hoxd11) and Pax2. The Hox11-Eya1-Pax2 complex binds to the Six2 enhancer to induce the expression of Six2, which, in turn, mediates Six2 and Gdnf activation (Gong et al., 2007). Thus, Eya1 acts as a key regulator of Gdnf expression and, hence, determination of metanephric fate within the intermediate mesoderm (Sajithlal et al., 2005).

Chicken ovalbumin upstream promoter-transcription factor I and II (COUP-TFI and COUP-TFII; Nr2f1 and Nr2f2, respectively – Mouse Genome Informatics) are members of the nuclear receptor superfamily (Qu et al., 1994). Although biochemical studies indicate that these two factors have similar DNA-binding and transcriptional activity in vitro, the patterns of COUP-TFI and COUP-TFII expression are distinct from each other. COUP-TFI is expressed in the mesenchyme of developing organs and is shown to play a key role in their organogenesis, cell fate determination, cell differentiation, angiogenesis and metabolic homeostasis (Kim et al., 2009; Kurihara et al., 2007; Li et al., 2009; Lin et al., 2010; Qin et al., 2010; Qin et al., 2008; Tang et al., 2010; Xie et al., 2011; You et al., 2005a; You et al., 2005b). During kidney development, COUP-TFII expression is detectable in the condensed mesenchyme and...
COUP-TFII in early nephrogenesis

Handover: COUP-TFII in early nephrogenesis

siRNA are described in supplementary material Table S1. RNA interference

RNA interference and quantitative real-time RT-PCR assay

The rat inducible metanephric mesenchyme (RIMM-18) cell line was

Human embryonic kidney 293 (HEK 293) cells were transfected with

visualized by staining with anti-E-Cadherin (BD Biosciences, 1:200).

93). Therefore, our studies clearly indicate that COUP-TFII is a key early regulator of the formation of metanephric mesenchyme and the subsequent formation and differentiation of the kidney.

MATERIALS AND METHODS

Animals

Generation of the floxed COUP-TFII mice and COUP-TFII-lacZ knock- in mice was as described previously (Takamoto et al., 2005). The Rosa26-Cre-ERT2+ knock-in mouse strain was provided by T. Ludwig (Columbia University, New York City, USA) (de Luca et al., 2005). For inducible deletions during embryonic stages, 2 mg tamoxifen (Tam), dissolved in corn oil (Sigma-Aldrich; 10 mg/ml), was injected intraperitoneally into pregnant females at E7.5 or E8.5. Embryos from littermates were collected at indicated stages. All mouse strains were maintained in a mixed genetic background and received standard rodent chow.

X-gal staining

Whole-mount X-gal staining of embryos (up to E10.5) was performed according to published methods (Takamoto et al., 2005).

Histology, immunofluorescence and in situ hybridization

All the histology and immunofluorescence staining of paraffin-embedded slides were performed as described (You et al., 2005a). Antibodies used were: anti-COUP-TFII (R&D, 1:2000), anti-Pax2 (Covance, 1:500), anti-Kit67 (BD Biosciences, 1:400), anti-β-galactosidase (Biogenesis, 1:1500), anti-Wil (Santa Cruz, 1:500), anti-Gdnf (Santa Cruz, 1:200) and anti-Six2 (ProteinTech group, 1:400). Non-radioactive in situ hybridization was carried out as described previously (Bramblett et al., 2004). The RNA probe for mouse Osr1 was provided by Thomas M. Schultheiss (Harvard University, Boston, USA) and the mouse Eya1 probe was as described (Xu et al., 1999). Cell apoptosis was detected by cleaved Caspase 3 (Cell Signaling, 1:500) staining or by TUNEL assay (Roche, In Situ Cell Death Detection Kit) according to the manufacturer’s instructions.

Kidney organ culture and staining

The metanephric mesenchymes containing the Wolffian duct from 10.5 days post-coitum (dpc) mouse embryos were cultured on transwell filters as described (Shakya et al., 2005). The Wolffian duct and ureteric bud were

Mouse embryonic kidney 293 (HEK 293) cells were transfected with

detectable in the distal tubules, podocytes and epithelial cells of Bowman’s capsule (Suh et al., 2006). Nonetheless, the expression pattern of COUP-TFII in kidney precursor cells has not been determined. Here, we employed a COUP-TFII-specific antibody to investigate its expression pattern in the nephrogenic mesoderm from E9.5 to E10 (Fig. 1A-C). At E9.5, COUP-TFII staining was observed in the intermediate mesoderm and other mesenchyme cells surrounding the Wolffian duct (Fig. 1A). Using Pax2 to mark the Wolffian duct, we demonstrated that COUP-TFII is only expressed in the intermediate mesoderm and not in the duct (Fig. 1B). Half a day later, at E10, COUP-TFII expression was observed in the nephrogenic mesenchyme region surrounding the Wolffian duct and in the urogenital ridge (Fig. 1C). These results indicate that COUP-TFII is expressed in kidney precursor cells.

Inducible deletion of COUP-TFII in kidney precursor cells

In order to bypass early embryonic lethality of COUP-TFII homozygous mutant at E10.5, we conditionally inactivated COUP-TFII by crossing the COUP-TFII floxed mouse strain (COUP-TFIIflx) (Takamoto et al., 2005) with Rosa26-Cre-ERT2+/+, a strain that harbors a tamoxifen-inducible Cre recombinase under the control of the ubiquitously active ROSA26 promoter (de Luca et al., 2005). Rosa26-Cre-ER2+/COUP-TFIIflx males (hereafter referred to as COUP-TFIIflx) were intercrossed with COUP-TFIIflx females, and a single dosage of 2 mg Tam was injected intraperitoneally into pregnant dams to induce COUP-TFII deletion. Specifically, we performed Tam injection at E7.5. In our mouse model, a lacZ gene inserted into the COUP-TFII locus is turned on when the COUP-TFII gene is deleted. One and half a days after Tam treatment, COUP-TFII was deleted throughout the whole body of COUP-
COUP-TFII is essential for metanephric mesenchyme formation but not for the formation of mesonephros

During the formation of the metanephros, the metanephric mesenchyme appears morphologically as an aggregate of the nephrogenic mesenchyme cells at the caudal end of the nephrogenic cord at E10.5 (Fig. 2A,C). This structure is largely missing in COUP-TFII<sup>id</sup> mutant embryos (Fig. 2B,D). In addition, serial sections through the entire metanephric mesenchyme region were examined with intermediates of the same somite number. The results clearly show that the nephrogenic mesenchyme is not formed in the COUP-TFII<sup>id</sup> mutant (supplementary material Fig. S1). As the paired box-containing transcription factor Pax2 is a key regulator of kidney development (Torres et al., 1995), we investigated whether the expression of this gene is compromised. In the mouse kidney, the expression of Pax2 is initiated in the intermediate mesoderm and the expression of this gene is compromised. In the mouse kidney, the expression of Pax2 is initiated in the intermediate mesoderm and the expression of this gene is compromised. However, the Pax2 expression in the Wolffian duct remains (F). The Pax2 expression in the Wolffian duct and condensed metanephric mesenchyme (mm) are seen in the COUP-TFII<sup>id</sup> control (A,C). The metanephric mesenchyme of COUP-TFII<sup>id</sup> control embryos expresses Pax2, Six2 and Gdnf (E,G,I). By contrast, these three gene products are undetectable in the metanephric mesenchyme region of COUP-TFII<sup>id</sup> null mutants (F,H,J). However, the Pax2 expression in the Wolffian duct remains (F). lacZ staining indicates that COUP-TFII is deleted in the mesenchyme cells (F). Boxed areas in I and J are shown in insets. In the kidney organ cultures from the metanephric mesenchyme (K) and COUP-TFII<sup>id</sup> mutant (L) embryos indicated that the ureteric bud (ub) outgrowth, visualized by E-cadherin staining, was detected from the Wolffian duct of the COUP-TFII<sup>id</sup> control (K) and COUP-TFII<sup>id</sup> mutant (L) embryos. DAPI counterstain for nuclei (blue) was applied for E-J. Scale bars: 50μm.

Development 139 (13) 2332 RESEARCH ARTICLE
is totally lost in the nephrogenic mesenchyme area, whereas it is intact in the Wolffian duct of the \( \text{COUP-TFI}^{d/d} \) mutants (Fig. 2F). \( \text{lacZ} \)-positive cells, which denote COUP-TFIi ‘expressing’ cells in the \( \text{COUP-TFI}^{d/d} \) mutants, indicated that some ‘COUP-TFIi expressing’ metanephric mesenchyme cells surrounding the Wolffian duct are still present (Fig. 2F).

In addition to Pax2, the homeobox gene Six2 is also specifically expressed in the metanephric mesenchyme before ureteric bud outgrowth (Xu et al., 1999) and is required to activate Gdnf expression by directly binding to its promoter during kidney development (Brodbeck et al., 2004). Therefore, we examined Six2 expression in COUP-TFIi mutants. As indicated in Fig. 2G,H, Six2 expression is not detectable in the mutant metanephric mesenchyme. As both Pax2 and Six2 lie upstream of expression, COUP-TFIi expression in COUP-TFIi mutants. As indicated in Fig. 2G,H, Six2 expression is not detectable in the mutant metanephric mesenchyme. As both Pax2 and Six2 lie upstream of expression, COUP-TFIi expression is not detectable in the mutant metanephric mesenchyme. As both Pax2 and Six2 lie upstream of expression, COUP-TFIi expression is not detectable in the mutant metanephric mesenchyme.

Therefore, we examined Six2 expression by directly binding to its promoter during kidney outgrowth (Xu et al., 1999) and is required to activate Gdnf expression by directly binding to its promoter during kidney development (Brodbeck et al., 2004). Therefore, we examined Six2 expression in COUP-TFIi mutants. As indicated in Fig. 2G,H, Six2 expression is not detectable in the mutant metanephric mesenchyme. As both Pax2 and Six2 lie upstream of expression, COUP-TFIi expression is not detectable in the mutant metanephric mesenchyme. As both Pax2 and Six2 lie upstream of expression, COUP-TFIi expression is not detectable in the mutant metanephric mesenchyme.

During this stage, the early mesonephros structure also formed. The mesonephros develops when the Wolffian duct reaches the prospective mesonephric mesenchyme and induces adjacent mesenchymal cells to condense and form mesonephric tubules (Saxén, 1987). First, we investigated whether COUP-TFIi is also expressed in the mesonephros (also called the mesonephric tubule). COUP-TFIi staining in E10 embryos indicated that COUP-TFIi is expressed in the mesonephros but not in the Wolffian duct (supplementary material Fig. S2A). Pax2 is expressed in the Wolffian duct and mesonephros. By using Pax2 as a mesonephric mesenchyme marker, we found COUP-TFIi colocalizes with Pax2 in the mesonephros but not in the Wolffian duct (supplementary material Fig. S2B). We examined the E9.5 embryos and found that the mesonephros is still intact in the \( \text{COUP-TFI}^{-/-} \) mutants in which COUP-TFIi is deleted very early in the germ cells, compared with \( \text{COUP-TFI}^{f/f} \) controls (supplementary material Fig. S2C,D). Therefore, even though COUP-TFIi is expressed in the mesonephros, it is not essential for mesonephros formation.

**COUP-TFIi specifically regulates Eya1, Wt1 and Six2 in the metanephric mesenchyme**

Although the metanephric mesenchyme markers, such as Pax2 or Six2 expression, are not detected in the \( \text{COUP-TFI}^{d/d} \) mutant embryos (Fig. 3F,H), the presence of \( \text{lacZ} \)-positive cells indicates that it is still possible that the lack of marker gene expression could be due to the loss of metanephric mesenchyme cells. To demonstrate that COUP-TFIi does indeed regulate metanephric mesenchyme marker gene expression, we deleted COUP-TFIi one day later by injecting Tam at E8.5 and collecting embryos at E10.5. We reasoned that by deleting COUP-TFIi one day later, we would have metanephric mesenchyme formation, but in some cells COUP-TFIi would be completely deleted, in some one allele would be deleted.
and in some it would not be deleted at all. In this scenario, a decrease in overall expression of these marker genes per cell would be observed in the formed metanephric mesenchyme. In addition, those cells with COUP-TFI deletion would have lower levels of target gene expression and those cells with no COUP-TFI deletion would have higher levels of target gene expression. As expected, we found that the metanephric mesenchyme was formed in both COUP-TFI control (Fig. 3A) and COUP-TFI d/d mutant (Fig. 3B) embryos, even though the mutant metanephric mesenchyme is smaller and less condensed compared with the control. Using in situ hybridization to examine Eya1 expression, we found that both the number of cells expressing Eya1 and the expression level per cell are decreased in the COUP-TFI d/d mutant (Fig. 3C,D). Similarly, Wt1 and Six2 expression levels in the metanephric mesenchyme are significantly decreased in the COUP-TFI d/d mutant (Fig. 3F,H) compared with the controls (Fig. 3E,G). To define further whether COUP-TFI specifically regulated Six2 expression in the metanephric mesenchyme, we double labeled Six2 with lacZ and found that Six2 colocalizes with lacZ expression in the metanephric mesenchyme of COUP-TFI d/d mutant (Fig. 3I). Under high magnification, we examined the Six2-lacZ-positive cells (Fig. 3J) and found that those cells with a higher lacZ signal (i.e. in which COUP-TFI is deleted) have lower Six2 expression (Fig. 3K,L, arrowheads). By contrast, if lacZ expression is low (i.e. COUP-TFI is not deleted), Six2 expression is higher (Fig. 3K,L, arrows).

In order to quantify the results, we measured the fluorescence intensity of Six2-positive cells (metanephric mesenchyme cells) in the COUP-TFI control (n=131 cells) and COUP-TFI d/d mutants (n=65 cells) shown in Fig. 3G,H and divided by the cell area to obtain densitometric levels of Six2 expression. The result shows that deletion of COUP-TFI significantly decreases Six2 expression in the COUP-TFI d/d mutant (Fig. 3M). These data clearly indicate that COUP-TFI does indeed regulate the expression of the metanephric mesenchyme markers Six2, Eya1 and Wt1.

**Deletion of COUP-TFI abolishes Wt1 expression and increases metanephric mesenchyme cell apoptosis**

We observed a reduction in cell number (Fig. 4A,B) in the nephrogenic mesenchyme region of the COUP-TFI d/d mutant embryos compared with controls. We investigated whether the loss of mesenchymal cells in this region is due to decreased cell proliferation or increased apoptosis. Embryo-wide comparison of Ki67 staining revealed no obvious differences in overall proliferation in controls versus COUP-TFI d/d mutants (Fig. 4A,B). By contrast, the COUP-TFI d/d mutant metanephric mesenchyme region showed a significant decrease in Ki67-positive cell numbers (Fig. 4C,D, red circled regions), indicating a specific decrease in proliferation of the metanephric mesenchyme. Furthermore, we observed an increase in the number of apoptotic cells in the metanephric mesenchyme of mutant mice as detected by both cleaved Caspase 3 and tunnel assays (Fig. 4E-H).

The Wilms tumor suppressor gene (Wt1) is expressed in the metanephric mesenchyme and has been shown to be essential for the survival of metanephric mesenchymal cells (Kreidberg et al., 1993; Kuure et al., 2000; Moore et al., 1999). Therefore, we investigated whether Wt1 expression is compromised in COUP-TFI d/d mutants. First, we determined whether COUP-TFI and Wt1 colocalize with each other. As shown in supplementary material Fig. S3A-C, we found that COUP-TFI colocalizes with Wt1 in the metanephric mesenchyme and in the urogenital ridge. Next, we investigated whether Wt1 expression level is altered with COUP-TFI deletion. Indeed, we found Wt1 expression to be significantly decreased in the COUP-TFI mutant urogenital ridge and metanephric mesenchyme (deletion cells shown as the lacZ positive cells) (Fig. 4E,F and 4I,J). These results indicate that Wt1 lies downstream of COUP-TFI and is regulated by COUP-TFI. Owing to the importance of Wt1 in metanephric mesenchyme cell survival, our findings suggested that loss of Wt1 expression due to COUP-TFI deletion is the likely reason for the increased metanephric mesenchyme apoptosis in COUP-TFI d/d mutants.

**COUP-TFs regulate the expression of Eya1, Six2, Wt1 and Gdnf in metanephric mesenchyme cells**

In order to study the detailed molecular mechanism by which COUP-TFI controls regulatory genes in the metanephric mesenchyme, we employed the conditionally immortalized rat induced metanephric mesenchyme cell line (RIMM-18). RIMM-18 cells were generated from rat mesenchymal cells by transfection with a vector encoding an estradiol-dependent E1A-ER fusion protein and...
COUP-TFII mutants by in situ hybridization. TFII is upstream of Osr1 by examining the expression of TFII in the regulatory cascade, we first investigated whether COUP-TFII acts as a positive regulator, enhancing target gene expression by interacting with Sp1 at Sp1-binding sites (Kim et al., 2009; Lin et al., 2010; Pipaon et al., 1999; Qin et al., 2010). To assess whether COUP-TFII regulates Eya1 expression through its interaction with Sp1, we searched for the evolutionarily conserved Sp1 sites of the Eya1 promoter and found three conserved sites (Fig. 6A,B). This indicates that Eya1 is a COUP-TFII target and is likely to be regulated by COUP-TFII at the transcriptional level.

To support this conclusion further, we carried out chromatin immunoprecipitation (ChIP) assays to determine whether COUP-TFII is recruited to the promoter/enhancer region of the Eya1 gene locus. Our laboratories have previously demonstrated that COUP-TFII acts as a positive regulator, enhancing target gene expression by interacting with Sp1 at Sp1-binding sites (Kim et al., 2009; Lin et al., 2010; Pipaon et al., 1999; Qin et al., 2010). To assess whether COUP-TFII regulates Eya1 expression through its interaction with Sp1, we searched for the evolutionarily conserved Sp1-binding sites in human, mouse and rat sequences on and surrounding the Eya1 gene and found three conserved sites upstream of the transcription start site (Fig. 6C, black boxes). We then performed ChIP assays to evaluate whether endogenous COUP-TFII is recruited to the Sp1 sites of the Eya1 promoter. Indeed, we found COUP-TFII is preferentially recruited to the promoter regions containing Sp1-binding sites but not recruited to the region lacking Sp1-binding sites (Fig. 6C, Sp1). In parallel, Sp1 was also specifically recruited to the same region as COUP-TFII in endogenous conditions (Fig. 6D, E).

**COUP-TFII directly regulates Eya1 gene expression by interacting with Sp1**

Eya1 has been shown to specify the metanephric mesenchyme fate and is indispensable for metanephric mesenchyme formation. In order to determine whether Eya1 expression is regulated by COUP-TFII at the transcriptional level, we carried out in situ hybridization to see whether Eya1 is regulated by COUP-TFII at the mRNA level. Indeed, Eya1 mRNA is detected in the metanephric mesenchyme of COUP-TFII[+/−] controls but is absent in COUP-TFII[−/−] mutants (Fig. 6A,B). This indicates that Eya1 is a COUP-TFII target and is likely to be regulated by COUP-TFII at the transcriptional level.

To support this conclusion further, we carried out chromatin immunoprecipitation (ChIP) assays to determine whether COUP-TFII is recruited to the promoter/enhancer region of the Eya1 gene locus. Our laboratories have previously demonstrated that COUP-TFII acts as a positive regulator, enhancing target gene expression by interacting with Sp1 at Sp1-binding sites (Kim et al., 2009; Lin et al., 2010; Pipaon et al., 1999; Qin et al., 2010). To assess whether COUP-TFII regulates Eya1 expression through its interaction with Sp1, we searched for the evolutionarily conserved Sp1-binding sites in human, mouse and rat sequences on and surrounding the Eya1 gene and found three conserved sites upstream of the transcription start site (Fig. 6C, black boxes). We then performed ChIP assays to evaluate whether endogenous COUP-TFII is recruited to the Sp1 sites of the Eya1 promoter. Indeed, we found COUP-TFII is preferentially recruited to the promoter regions containing Sp1-binding sites but not recruited to the region lacking Sp1-binding sites (Fig. 6C, Sp1). In parallel, Sp1 was also specifically recruited to the same region as COUP-TFII in endogenous conditions (Fig. 6D, E).

**COUP-TFII signaling is independent of Osr1 in the nephrogenic mesenchyme**

Similar to COUP-TFII, Odd-skipped related 1 (Osr1) is also required for the formation of the metanephric mesenchyme and is upstream of Eya1. In order to define the relationship between Osr1 and COUP-TFII in the regulatory cascade, we first investigated whether COUP-TFII is upstream of Osr1 by examining the expression of Osr1 in COUP-TFII mutants by in situ hybridization. Osr1 is broadly expressed in the nephrogenic mesenchyme regions in the control mice (supplementary material Fig. S5A). Despite the fact that the COUP-TFII[−/−] mutant has fewer mesenchyme cells, the Osr1 expression level in the mesenchyme is equivalent to that of the control (supplementary material Fig. S5B). Next, we examined COUP-TFII expression in Osr1 knockout mice to determine whether COUP-TFII is downstream of Osr1. The COUP-TFII expression level is similar in the urogenital ridge of Osr1 knockout and wild-type mice (supplementary material Fig. S5C-F). Although Osr1 knockouts lack the metanephric mesenchyme, COUP-TFII expression in the nephrogenic mesenchyme surrounding the Wolffian duct is similar compared with wild-type controls (supplementary material Fig. S5C-F). These results indicate that Osr1 and COUP-TFII act in parallel with one another and are both required for Eya1 activation to control metanephric mesenchyme differentiation and nephrogenesis. To support this conclusion further, we employed RIMM-18 cell culture experiments and found that simultaneous knockdown of COUP-TFII and COUP-TFII by siRNA does not affect expression of Osr1 (supplementary material Fig. S5G-I). Similarly, knockdown of Osr1 had no significant effect on COUP-TFII or COUP-TFII expression (supplementary material Fig. S5J-L). These results support the conclusion that COUP-TFII and Osr1 act in parallel to regulate Eya1 expression.

These cells maintain its metanephric mesenchyme characteristics (Levashova et al., 2003). Using RT-PCR, we found that the expression levels of both COUP-TFI and COUP-TFII are high in RIMM-18 cells. Despite this, in the E10.5 mouse embryos, only COUP-TFII is expressed in the metanephric mesenchyme, whereas COUP-TFI is located in the stroma mesenchyme. Our laboratory has reported that COUP-TFI and COUP-TFII are highly homologous and also functionally redundant (Tsai and Tsai, 1997; Wu et al., 2010). Therefore, during in vitro experiments, we used siRNAs to knock down both COUP-TFI and COUP-TFII to study the underlying mechanism of COUP-TF regulation of transcription factors important for kidney development. Knockdown of COUP-TFI and COUP-TFII significantly decreases Gdnf, Eya1, Six2 and Wt1 (Fig. 5C-F) expression. In order to confirm whether COUP-TFII alone can regulate these transcription factors in RIMM-18 cells, we also used siRNA to knock down COUP-TFII specifically. We found that knockdown of COUP-TFII alone is enough to significantly decrease Eya1 and Wt1 (supplementary material Fig. S4B,C) expression. These results indicate that COUP-TFII can indeed positively regulate genes expressed early in metanephric mesenchyme development.

**COUP-TFII signaling is independent of Osr1 in the nephrogenic mesenchyme**

Similar to COUP-TFII, Odd-skipped related 1 (Osr1) is also required for the formation of the metanephric mesenchyme and is upstream of Eya1. In order to define the relationship between Osr1 and COUP-TFII in the regulatory cascade, we first investigated whether COUP-TFII is upstream of Osr1 by examining the expression of Osr1 in COUP-TFII mutants by in situ hybridization. Osr1 is broadly expressed in the nephrogenic mesenchyme regions in the control mice (supplementary material Fig. S5A). Despite the fact that the
When COUP-TFII was expressed compared with control cells (Fig. 5F). Next, we mutated these three conserved Sp1-binding sites as depicted in supplementary material Fig. S4A. We generated reporters with three single Sp1-binding sites or with all three sites mutated (supplementary material Fig. S6A; pGL2-Eya1-M1, pGL2-Eya1-M2, pGL2-M3 and the triple-mutation pGL2-Eya1-M123). In the presence of COUP-TFII expression plasmid, activation of all four mutant luciferase reporters was significantly diminished versus the intact reporter (Fig. 6F). This result indicates that COUP-TFII works through Sp1 on the Sp1-binding sites to activate Eya1 expression.

Collectively, these results substantiate a model in which COUP-TFII is recruited to the Eya1 promoter in an Sp1-dependent manner to directly activate Eya1 transcription.

**COUP-TFII interacts synergistically with Sp1 to regulate Wt1 to modulate Gdnf expression during metanephric induction**

Next, we investigated whether COUP-TFII also directly regulates the transcription of Wt1 through its interactions with Sp1. We identified two evolutionarily conserved Sp1-binding sites surrounding the Wt1 promoter (Fig. 7A, black boxes). ChIP analysis showed that COUP-TFII is indeed preferentially recruited to Sp1-binding sites at the Wt1 promoter but is not recruited to the region lacking Sp1-binding sites (Fig. 7A). Sp1 was recruited to the same regions as COUP-TFII. To determine whether recruitment of COUP-TFII to the Wt1 promoter is Sp1-dependent, we knocked down endogenous Sp1 by siRNA. With knockdown, COUP-TFII recruitment to the Wt1 promoter was significantly reduced (Fig. 7B, C).

To confirm whether COUP-TFII binding to the Wt1 promoter leads to transcription activation, we performed luciferase reporter assays using a 1.1-kb (–736 bp to 377 bp) human WT1 promoter fragment that contains the two conserved Sp1-binding sites. As shown in Fig. 7D, Wt1-luciferase reporter (pGL2-Wt1) activity was significantly increased by COUP-TFII. In addition, when Sp1-binding sites were mutated in the Wt1-luciferase reporter (supplementary material Fig. S6B; pGL2-Wt1-M1, pGL2-Wt1-M2, pGL2-Wt1-M3 and pGL2-Wt1-M123 (250 ng each) were measured after co-transfection with or without COUP-TFII expression plasmid (50 ng) into HEK 293 cells.

**DISCUSSION**

COUP-TFII directly regulates transcription of Eya1 to modulate Gdnf expression during metanephric induction

COUP-TFII is expressed in the mesenchyme of developing organs and has been shown to play a key role in their organogenesis. In this study, we found that COUP-TFII is expressed in the kidney
suggesting that COUP-TFII directly regulates Eya1 expression. Furthermore, expression of COUP-TFII can enhance Eya1 expression and, thus, induces metanephric mesenchyme formation. These results place COUP-TFII as an upstream regulator of these important regulatory factors.

Our in situ hybridization data showed that Eya1 expression is significantly decreased in the COUP-TFII mutant (Fig. 3C,D), suggesting that COUP-TFII directly regulates Eya1 expression in the metanephric mesenchyme. Indeed, ChIP assays showed that COUP-TFII is recruited to the conserved Sp1-binding site of the Eya1 promoter by tethering to Sp1 for direct regulation of Eya1 expression. Furthermore, expression of COUP-TFII can enhance Eya1 promoter-driven reporter activity. Collectively, these results clearly indicate that COUP-TFII promotes Gdnf signaling cascade by direct regulation of Eya1 expression and, thus, induces metanephric mesenchyme fate in kidney progenitor cells. It should be noted that in Eya1 knockout mice, ureteric buds fail to grow out into the metanephric mesenchyme (Sajithlal et al., 2005). Similar to Eya1, we observed no ureteric bud outgrowth in six out of eight COUP-TFII mutants using the kidney organ cultures (Fig. 2L). It is not clear, however, whether the remaining two COUP-TFII mutants have ureteric bud outgrowth (supplementary material S7A,B, arrowheads). In any event, if they indeed have ureteric bud outgrowth, it is possible that COUP-TFII was not deleted early enough in these two particular mutants, resulting in partially committed metanephric mesenchyme and subsequent induction of ureteric bud outgrowth. As the majority of mutants do not have ureteric buds outgrowth, it indicates that COUP-TFII is required for the proper formation of metanephric mesenchyme and subsequent induction of ureteric bud outgrowth.

COUP-TFII acts in parallel with Osr1 to regulate Eya1 expression during metanephric induction

Osr1 is known to be the earliest marker for kidney development in the intermediate mesoderm. Mice lacking Osr1 do not form metanephric mesenchyme and do not express many factors essential for metanephric mesenchyme formation (James et al., 2006; Wang et al., 2005). These phenotypes bear strong resemblance to that of the COUP-TFII knockout mice. To address whether these two genes work in the same pathway, we examined whether Osr1 expression is affected in the COUP-TFII knockout. Our results clearly show that Osr1 expression in the nephrogenic mesenchyme region is not altered in the COUP-TFII mutant. Similarly, COUP-TFII expression in the metanephric mesenchyme region remains the same in the Osr1 knockout embryo. Together, these results indicate that COUP-TFII and Osr1 act independently to regulate kidney morphogenesis. This notion is further supported by in vitro cell culture experiments, in which we showed that COUP-TFII and Osr1 do not regulate each other’s expression. Interestingly, whereas Wt1 is expressed in the metanephric mesenchyme of Osr1 null mice (James et al., 2006), Wt1 expression in the COUP-TFII knockout mutant is totally lost. This result indicates that although both COUP-TFII and Osr1 are expressed in the intermediate mesoderm and both regulate Eya1, Pax2 and Six2 expression, they do not regulate each other to control the expression of those key factors important for metanephric mesenchyme formation. Based on all these results, our working model is that COUP-TFII and Osr1 act in parallel to regulate Eya1 and its downstream target Gdnf to specify metanephric mesenchyme (Fig. 8).

COUP-TFII is essential for survival of the metanephric mesenchyme and for nephron differentiation

Wt1 mutant mesenchyme cannot be induced to form nephric tubules (Kreideberg et al., 1993), suggesting that Wt1 is cell-autonomously required for nephron differentiation. Subsequently,
Fig. 8. COUP-TFII directly regulates Eya1 and Wt1 expression to specify metanephric mesenchyme cell fate and maintain metanephric mesenchyme precursor cell survival. COUP-TFII acts in parallel with Osr1 to regulate Eya1 transcription. COUP-TFII in the intermediate mesoderm kidney precursor cells specifies metanephric mesenchyme fate by directly regulating Eya1 expression. Eya1 regulates Pax2 expression, forms a complex with Pax2 and Hox11 paralogous proteins (including Hoxa11, Hoxc11 and Hoxd11) and binds directly to the Six2 enhancer to regulate the expression of Six2 and its downstream target Gdnf. Gdnf, a crucial factor for the specification of the metanephric mesenchyme, will then induce ureteric bud outgrowth from the Wolffian duct and initiate kidney organogenesis. In addition, COUP-TFII is essential for the survival of the metanephric mesenchyme precursor cells through its direct regulation of Wt1 gene expression. Wt1 plays an anti-apoptotic role to maintain metanephric mesenchyme cell survival. MM, metanephric mesenchyme; UB, ureteric bud.

it was shown that kidney precursor cells undergo apoptosis in Wt1-deficient mutant mice, consistent with the notion that Wt1 is essential for the early stage of kidney development (Davies et al., 2004; Kreidberg et al., 1993). The colocalization of COUP-TFII and Wt1 in the metanephric mesenchyme and urogenital ridge and the similar phenotypes exhibited by COUP-TFII and Wt1 mutants in terms of decreased cell numbers in the metanephric mesenchyme strongly implicate that these two factors function in the same pathway. As the expression of Wt1 is drastically decreased in COUP-TFII mutant mice in the metanephric mesenchyme and urogenital ridge (Fig. 3E,F and Fig. 4I,J), it suggests that COUP-TFII upstream of Wt1 in the signaling cascade. This notion is supported by ChIP analysis, which showed that COUP-TFII is recruited by Sp1 to the conserved Sp1-binding site in the Wt1 promoter to regulate Wt1 transcription directly. Therefore, Wt1 mediates COUP-TFII function to maintain metanephric mesenchyme cell differentiation and survival (Fig. 8).

In summary, we have shown that COUP-TFII has two major roles during metanephric mesenchyme formation. First, COUP-TFII directly regulates Eya1 transcription to specify the kidney precursor cell differentiation into the mature metanephric mesenchyme. Second, COUP-TFII directly regulates Wt1 expression to maintain metanephric mesenchyme survival and differentiation into the mature nephron.

Acknowledgements
We thank Dr Thomas Ludwig for providing the ROSA26Cre-ERT2 mice, Dr Thomas M. Schultheiss for the mouse Osr1 probe, Dr Pin-Xian Xu for the Eya1 mouse probe and Dr Alan O. Perantoni for sharing R1M1-18 cells. We appreciate technical help from Ms Wei Qian, Xuefei Tong and Grace Wen Chen. We would like to express gratitude to Eric Buras for the English editing.

Funding
This work was supported by grants from the National Institutes of Health [DK62434 and DK59820 to S.Y.T. and M.-J.T., HL76448 to S.Y.T., DK45641 and HD17379 to M.-J.T. and the Diabetes Endocrinology Research Center P30 DK079638 to M.-J.T.]. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.076299/-/DC1

References
Li, L., Xie, X., Qin, J., Jeha, G. S., Saha, P. K., Yan, J., Haueter, C. M., Chan, L., Tsai, S. Y. and Tsai, M. J. (2009). The nuclear orphan receptor COUP-TFII plays an essential role in adipogenesis, glucose homeostasis, and energy metabolism. Cell Metab. 9, 77-87.
COUP-TFII in early nephrogenesis

RESEARCH ARTICLE 2339