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Etv4 and Etv5 are required downstream of GDNF and Ret for kidney branching morphogenesis

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Abstract

GDNF signaling through the Ret receptor tyrosine kinase is critical for ureteric bud branching morphogenesis during kidney development, yet few of the downstream genes are currently known. We find that the ETS transcription factors Etv4 and Etv5 are positively regulated by Ret signaling in the ureteric bud tips. *Etv4*–/–, *Etv5*+/– mice display either renal agenesis or severe hypodysplasia, while kidney development fails completely in double homozygotes. We identify several genes whose expression in the ureteric bud depends on *Etv4* and *Etv5*, including *Cxcr4*, *Myb*, *Met, Mmp14*. Thus, *Etv4* and *Etv5* are key components of a gene network downstream of *Ret* that promotes and controls renal branching morphogenesis.

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Author Contributions

BL, CC, XC, and SK designed, performed and interpreted experiments. RK performed experiments. CB, MH, SJ, NA and MT contributed mutant kidneys. SA, LM and JH generated the *Etv5-lacZ* and *Etv4-lacZ* mice. KS-O and JB assisted with microarray analyses. VD'A analyzed renal histopathology. FC conceived and directed the project and wrote the manuscript. All authors edited the manuscript.

Competing Financial Interests

The authors have no competing financial interests.

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Introduction

During kidney development, the ureteric bud (UB), a tubular outgrowth of the Wolffian duct (WD), develops into the epithelium of the urinary collecting system through a complex process of branching morphogenesis. These events are controlled largely by signals to the UB cells from an adjacent population of mesenchymal cells, the "metanephric mesenchyme" (MM), and several of the responsible growth factors and their receptors have been identified[1–3]. In a reciprocal inductive process, factors secreted by the UB induce progenitor cells in the MM to condense, epithelialize and differentiate into nephrons, the functional units of the kidney[2–4]. Defects in UB branching morphogenesis can lead to reduced nephron number, which has been linked to the progression of renal diseases and hypertension[5].

One secreted protein that plays a crucial role in UB growth and branching is glial cell line-derived neurotrophic factor (GDNF), which is produced by the MM cells and signals via the receptor tyrosine kinase Ret and the co-receptor Gfrα1[6], which are co-expressed in the Wolffian duct and later at the tips of the UB. The ablation of any of these genes in mice results in absence of the kidney (agenesis), due to failure of UB outgrowth, or severe malformation (hypodysplasia) due to very limited UB branching[7]. In humans, *RET* mutations are associated with renal agenesis as well as Hirschsprung disease and cancer[6,8]. While the consequences of GDNF signaling include UB cell proliferation, cell survival, and branching of the epithelium, the precise mechanisms by which GDNF exerts its effects on UB cells remain to be elucidated[7].

Some of the genes regulated by GDNF are likely to promote cell proliferation, movement, adhesion, or shape changes, and thus contribute to morphogenesis of the epithelium, while others may encode inductive proteins secreted by the UB, or promote collecting duct cell differentiation. Only a few genes regulated by GDNF have been identified, and none of them can account for the profound effects of GDNF on UB morphogenesis. For this reason, we performed a genome-wide analysis of mRNA expression in the isolated UB cultured with or without GDNF. Among the genes identified were two members of the Pea3 family of ETS transcription factors[9], *Etv4* (*Pea3*) and *Etv5* (*Erm*), and here we focus on their roles in kidney development. *Etv4* and *Etv5* are known to be important in processes including neuronal, spermatogonial and limb development[10–12], but a role in renal development had not been investigated. We show that the overlapping expression of *Etv4* and *Etv5* in UB tip cells is positively regulated by GDNF/Ret signaling, and that these transcription factors are jointly required for kidney development. Thus, mice lacking both *Etv4* alleles and one *Etv5* allele have a high frequency of renal agenesis or hypodysplasia, due to branching defects, while in mice completely deficient in *Etv4* and *Etv5,* kidney development fails entirely. We next identify several genes whose expression in the UB tips depends on *Etv4* and *Etv5*, and which together may contribute to the defects seen in *Etv4*/*Etv5* as well as *Gdnf*, *Ret* or *Gfra1* mutant kidneys. These studies begin to elucidate a gene regulatory network activated by GDNF that controls ureteric bud branching morphogenesis.

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Results

A screen for genes whose expression in the ureteric bud is altered by GDNF

To identify genes whose expression is altered by GDNF/Ret signaling, we took advantage of the ability of isolated UBs to grow and branch in the absence of mesenchyme, when cultured in GDNF-supplemented Matrigel13. The mesenchyme was removed from E11.5 metanephroi, and the ureteric buds were cultured overnight +/−GDNF (Fig. 1a). Without GDNF, the UBs do not grow or branch appreciably13, but after 16 hours remain viable and capable of resuming growth if GDNF is added.

We surveyed Affymetrix murine gene chips U74Av2 and 430A using RNA from three pools of UBs grown with, and two without, GDNF. Of the ~12,000 transcripts represented on the U74Av2 array, 89 were upregulated by ≥2-fold with GDNF, 14 by ≥3-fold, and four by ≥4-fold, while only three were downregulated ≥2-fold. Of the 22,626 transcripts represented on the 430A array, 78 were upregulated by >2-fold, six by >3-fold, and three by >4-fold, and four were downregulated by ≥2-fold.

Table 1 lists the genes most highly upregulated by GDNF (more extensive lists are provided in Supplementary Tables 1 and 2). The validity of the screen was supported, first, by the fact that eight of these genes had been independently identified as GDNF/Ret targets in the UB (*Wnt11*, *Ret*, *Spry1*) or in other cell types (*Etv4*, *Etv5, Cxcr4, Ccnd1, Dusp6*)10,14–20. A second criterion was that genes upregulated by GDNF should be more highly expressed in the UB tip, the subdomain of epithelium where *Ret* and *Gfrα1* are expressed, than in the trunk. Twelve of the listed genes were examined by *in situ* hybridization (ISH), and all displayed tip-specific expression in the UB (Fig. 1b–h, Fig. 2i–j, and data not shown).

A third criterion for positive regulation by GDNF was reduced expression in a hypomorphic *Ret* mutant, in which GDNF/Ret signaling is reduced15. We examined the expression of nine GDNF-upregulated genes in *Ret*-hypomorphic kidneys, and all were significantly reduced in the mutant UB tips (Fig. 1b–h, Fig. 3c–f).

The genes we found to be upregulated by GDNF encode secreted factors (*Wnt11*, *Crlf1*), receptors (*Cxcr4, Ret*), regulators of signal transduction (*Spry1, Dusp6, Spred2*), transcription factors (*Etv5, Myb, E2f8, Etv4*), and enzymes (*Arg2, Car2)*. Several are associated with DNA replication, mitosis and cell cycle regulation (e.g., *Myb*, *Ect2*, *Ccnd1*, *Ccnb1, Ncaph*), consistent with a role of GDNF in proliferation of UB tip cells14,21. We hypothesize that these genes together mediate the effects of GDNF on branching morphogenesis, and that mutations in some of them may reveal roles in kidney development. Only *Ret*, *Wnt11* and *Spry1* are known to be required for normal UB branching morphogenesis16,22,23, while most of the others have been knocked out but caused no reported renal defects, or caused early embryonic lethality (http://www.informatics.jax.org/). Therefore, more complex genetic approaches, such as double or conditional knockouts, may be required to reveal potential functions during kidney development.

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Etv4 and *Etv5* are regulated primarily by GDNF/Ret signaling in the developing ureteric bud

The observation that two closely related genes, *Etv4* and *Etv5*, were both identified in the GDNF screen, together with their roles in other developing systems, suggested that they might encode key transcription factors that mediate the effects of extracellular cues during kidney development. Both genes are expressed in several organs undergoing branching morphogenesis, including the lung, mammary and salivary glands, and kidney24. We surveyed their expression during kidney development, and found that both *Etv4* and *Etv5* are expressed in a pattern similar to *Ret*: in the Wolffian duct at E9.5 and E10.5 (Fig. 2a–d), in the T-shaped UB at E11.5 (Fig. 2e–h) and in the UB tips throughout subsequent development (Fig. 2i–k). Unlike *Ret*, they are also expressed in the MM and nascent nephrons, but at lower levels than in the UB (Fig. 2g–j). The third Pea3-family member, *Etv1*, was not detectably expressed in E14.5 and E18.5 kidneys (data not shown).

To further test whether *Etv4* and *Etv5* are regulated by GDNF, we performed gain- and loss-of-function studies. Culture of kidneys heterozygous for *Etv4-lacZ* or *Etv5-lacZ* alleles with GDNF-soaked beads caused elevated lacZ expression in the UB tips close to the beads (Fig. 3a–b), confirming upregulation by GDNF. Conversely, in *Ret*-hypomorphic kidneys (Fig. 3c–f), or normal kidneys cultured with GDNF-blocking antibody (Supplementary Fig. 1a–d), *Etv4* and *Etv5* expression was greatly reduced.

Etv4 and *Etv5* are regulated by FGFs in several developing organs12,25,26. The two FGFs implicated in UB branching *in vivo* are FGF7 and FGF10, which are expressed in the MM and stroma27 and signal to the UB, mainly via FGFR2. In *Fgf10*−/− kidneys, or in kidneys in which *Fgfr2* was deleted in the UB (*Fgfr2*$^{UB-/-}$), both of which are hypoplastic with reduced UB branching28,29 *Etv4* and *Etv5* expression remained normal (Supplementary Fig 2a–h). Thus, in the developing UB, the normal expression of *Etv4* and *Etv5* depends strongly on GDNF/Ret signaling but apparently not on FGF7 or FGF10 signaling via FGFR2, and the defects in *Fgf7*, *Fgf10* and *Fgfr2*$^{UB-/-}$ mutant kidneys are evidently not due to reduced *Etv4*/*Etv5* expression. However, *Etv4* and *Etv5* expression in the UB can be upregulated by exogenous FGF10 (but not FGF7), as shown by culturing kidneys with FGF-soaked beads (Supplementary Fig. 1e–f, Supplementary Fig. 2i–n).

Ret signaling activates several intracellular signal transduction pathways, including Erk MAP kinase, PI3-kinase, and PLCγ 6. To investigate the mechanism that lead to expression of *Etv4* and *Etv5*, we employed mutant mice as well as pharmacological inhibitors. Ret tyrosine-1015 (Y1015) is required for PLC-γ activation, and *Ret*^{Y1015F} mutants have decreased UB branching30. However, *Etv4* and *Etv5* were expressed normally in *Ret*$^{Y1015F/-}$ kidneys (Supplementary Fig. 3a–d), indicating that PLC-γ signaling is dispensable for their expression. Mutation of Ret tyrosine-1062 (Y1062) reduces (but does not eliminate) both Erk MAP kinase and PI3K signaling, and also causes reduced UB branching *in vivo*31. *Ret*^{Y1062F} mutant kidneys expressed *Etv4* and *Etv5* at normal levels (Supplementary Fig. 3e–h). As there is not a *Ret* mutation that specifically eliminates Erk or PI3K signaling, we employed specific chemical inhibitors of these pathways in wild type kidneys cultures. While each inhibitor substantially reduced UB branching, as previously reported32,33, the

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PI3K inhibitor eliminated *Etv4* and *Etv5* expression in the UB, while the Erk MAP kinase inhibitor had little or no effect on expression (Supplementary Fig. 3i–x). Thus, the PI3K pathway appears to be critical for expression of *Etv4* and *Etv5* in the UB tips.

Etv4 and *Etv5* are together required for normal kidney development

To test the importance of *Etv4* and *Etv5* in kidney development, we examined single and compound mutant fetal and newborn mice, initially using *Etv4-lacZ*10 and *Etv5-lacZ* knockin/knockout alleles, which delete exons encoding the DNA binding domains (Fig. 4a–h). While *Etv4*$^{+/-}$ and *Etv5*$^{+/-}$ heterozygotes were normal, *Etv4*$^{-/-}$ homozygotes and *Etv4*$^{+/-}$*;Etv5*$^{+/-}$ compound heterozygotes had occasional renal agenesis (2% and 8%, respectively) or hypoplasia (9% and 15%) (Supplementary Table 3a–b). *Etv5*$^{-/-}$ homozygotes died before kidney development begins, so the most severe compound mutant examined was *Etv4*$^{-/-}$*;Etv5*$^{+/-}$, and in these newborn mice nearly all of the kidneys were either absent (41%) (Fig. 4d, left side) or hypoplastic (52%) (Fig. 4d, right side) (Supplementary Table 3b), although the mice were grossly normal. The hypoplastic kidneys were not only small but were cystic, lacked a nephrogenic zone, and had greatly reduced tubular elements and glomeruli (Fig. 4f), but the few glomeruli were apparently normal (Fig. 4h). Therefore, *Etv4* and *Etv5* are functionally redundant, and collectively support kidney development.

We next examined a second *Etv5* allele11, *Etv5*^{tm1Kmm} (abbreviated *Etv5*^{M}), in which exons 2–5, including the initiation codon, were deleted (Fig. 4i–m). *Etv5*^{M} is apparently weaker than *Etv5-lacZ,* as *Etv5*$^{M/M}$ homozygotes are viable, and *Etv4*$^{-/-}$*;Etv5*$^{+/M}$ mutants had less frequent renal defects (e.g., Fig. 4j) than *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ (Supplementary Table 3b–c) (the reason for this difference is unclear, but *Etv5*^{M} could potentially encode a partially functional protein fragment including the DNA binding domain). The viability of *Etv5*$^{M/M}$ homozygotes allowed us to generate *Etv4*$^{-/-}$*;Etv5*$^{M/M}$ double homozygotes, and in these mutants kidney development failed completely. 6/7 newborn mice had bilateral renal agenesis, and only one had a single, tiny kidney rudiment (Fig. 4k, Supplementary Table 3c), which was disorganized and showed no signs of UB branching or nephrogenesis (Fig. 4m). Despite the differences in severity of the two *Etv5* alleles, their *qualitatively* similar effects (combined with *Etv4* mutation) leave no doubt that these two ETS transcription factors play crucial and overlapping roles in kidney development. Thus, our studies identify, for the first time, two genes that are targets of the GDNF/Ret pathway, and when mutated recapitulate the phenotype of *Gdnf*, *Ret* or *Gfra1* mutants.

Renal agenesis or hypoplasia results from defects in ureteric bud formation and branching

To examine the cause of the renal agenesis/hypoplasia in *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ mutants, we examined UB branching by introducing a *Hoxb7/myrVenus* transgene expressing a fluorescent protein in the WD and UB34. At E11.5-E12.5, when the UB has normally emerged and started to branch, 16% of UBs were absent, 40% showed no branching, and 19% had retarded branching (Fig. 5a–d, Supplementary Table 4). At later stages, kidneys were often absent (Fig. 5i–j), and when present contained a reduced number and irregular pattern of UB tips (Fig. 5g–h). Thus, defects in UB formation and branching appear to

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underlie the severe renal defects, consistent with a role for *Etv4* and *Etv5* downstream of GDNF/Ret signaling.

As *Etv4* and *Etv5* are expressed in MM as well as UB, a deficiency in one or both lineages might contribute to branching defects. To test their requirement in the UB lineage, we cultured mutant UBs, isolated free of MM, under conditions where wild types grow and branch extensively13. None of the $Etv4^{-/-};Etv5^{+/-}$ UBs (0/4) branched significantly (Fig. 5m), whereas the majority with only one or two mutant alleles branched extensively (11/17; e.g., **Fig. k–l**) ($p<0.05$, t-test). This confirms that *Etv4* and *Etv5* are together important in the UB lineage for growth and branching.

A survey of candidate genes identifies several that require *Etv4*/*Etv5* for normal expression in the ureteric bud

As Etv4 and Etv5 are transcription factors, we hypothesized that the renal defects in compound mutants result from a failure to regulate their target genes. We therefore examined the expression of a variety of candidate genes by ISH or immunofluorescence, using kidneys from $Etv4^{-/-};Etv5^{+/-}$ embryos (rather than $Etv^{-/-};Etv5^{M/M}$ double homozygotes, which had no renal tissue to analyze).

The expression levels of *Gdnf*, and several other genes with important roles in the metanephric mesenchyme (*Wt1, Six1, Six2, Eya1, Pax2*), were not significantly altered in the MM of hypoplastic $Etv4^{-/-};Etv5^{+/-}$ kidneys, nor were markers of nascent nephron epithelia (*Lim1*, *Jag1, Pax2*), podocytes (*Wt1, Lim1*), and stroma (*Foxd1*) (Supplementary Fig. 4). This is consistent with the morphologically normal glomerular differentiation in these mutant kidneys (Fig. 4h).

Several of the genes positively regulated by GDNF/Ret signaling in the UB tips (Fig. 1 and Table 1), were examined in the hypoplastic $Etv^{-/-};Etv5^{+/-}$ kidneys. At E15.5, *Crlf1, Dusp6*, *Etv5* and *Spry1* were expressed at close to normal levels (Fig. 6, data not shown). *Ret* remained highly expressed in many mutant UB tips, although it was low in a subset of the tips, while *Wnt11* was uniformly reduced. However, at E13.5, expression of *Ret* (not shown) and *Wnt11* (Fig. 6i) was normal, suggesting that their reduced expression at E15.5 is a secondary effect of the progressively abnormal renal development in $Etv4^{-/-};Etv5^{+/-}$ mutants. In contrast, expression of the chemokine receptor *Cxcr4* and the transcription factor *Myb* were strongly reduced in the mutant kidneys at both E13.5 and E15.5 (Fig. 6f,g,j,k), suggesting they are more likely to be primary targets of Etv4/Etv5 activity.

We also examined *Met* and *Mmp14*, which were not identified in our screen as GDNF targets (perhaps for technical or biological reasons, e.g., effects of Matrigel or culture medium on gene regulation, or low expression at E11.5), but are regulated via *Etv4* in other cell types and have been implicated in kidney development35–38. *Met,* the tyrosine kinase receptor for hepatocyte growth factor (HGF) is regulated by *Etv4* in motor neurons39, and is expressed in UB tips. *Met* mRNA was dramatically reduced in $Etv4^{-/-};Etv5^{+/-}$ kidneys at both E13.5 and E15.5 (Fig. 6h,l). Consistent with its regulation by *Etv4*, *Met* expression was also absent in the UB of *Ret*-hypomorphic kidneys (not shown). The matrix metalloprotease MMP14 is likely a direct target of *Etv4* in tumor cells40, and is implicated in branching

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morphogenesis in kidney and other organs36,41–43. In wild type kidneys, MMP14 protein was detected in the UB, MM and peripheral stroma (Fig. 7a,e), while in *Etv4*$^{-/-}$*;Etv5*$^{+/-}$, it was strongly reduced in both the UB and MM (Fig. 7c,g). Similarly, in *Ret*-hypomorphic kidneys, MMP14 was greatly diminished in both UB and MM (Fig. 7k). Since *Ret* is only expressed in the UB, the reduced expression of MMP14 in MM is likely an indirect effect of the *Ret* mutation. Consistent with this interpretation, Mmp14 mRNA is normally expressed in the UB and the stroma, but not the MM (Fig. 7m), and it is downregulated in the UB of *Ret*-hypomorphic kidneys (Fig. 7n); therefore, the MMP14 protein observed in the MM is probably shed from the UB, a known property of MMP1444. We conclude that *Mmp14* is normally expressed in the UB, where it is positively regulated by Ret signaling, via *Etv4* and *Etv5*, and in the stroma where it is independent of these mechanisms.

Discussion

To identify genes that mediate the important effects of Ret signaling on branching morphogenesis, we conducted a screen for genes whose expression is altered by GDNF in UB cultures. In addition to several previously described GDNF "targets" in the UB (*Ret*, *Wnt11*, *Spry1*) or in other cell types (*Etv4, Etv5*), we identified several new downstream genes including *Cxcr4*, *Crlf1*, *Myb* and *Dusp6.* We then focused on the roles of the transcription factors *Etv4* and *Etv5*, and found that they exhibit largely overlapping expression throughout kidney development, and that both are positively regulated by GDNF/Ret signaling in UB tips. Genetic crosses revealed that mice lacking three of the four *Etv4*/*Etv5* alleles have moderate to severe defects in renal development, due to reduced UB branching, while those lacking all four alleles fail to develop kidneys. We next identified four genes whose expression is greatly reduced in the hypoplastic kidneys of *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ compound mutants, and thus represent direct or indirect targets of these transcription factors. *Cxcr4*, *Met*, *Mmp14,* and *Myb* may participate in different aspects of epithelial morphogenesis, and collectively, their reduced expression likely contributes to the renal defects in *Etv4/Etv5* mutants as well as *Ret, Gdnf* or *Gfra1* mutant mice (Supplementary Fig. 5).

Etv4 and *Etv5* are transcriptionally regulated by FGFs in several organs12,25,26, and FGFRs activate several signaling pathways common to Ret45. Therefore, it was surprising that renal expression of *Etv4* and *Etv5* was unaffected by *Fgf10* or *Fgfr2* mutations. Most likely, the strong GDNF/Ret signaling in the UB overwhelms the effects of FGF signaling, so a reduction in the latter has no noticeable effect on *Etv4*/*Etv5* expression. However, *Etv4* and *Etv5* expression was increased in kidneys cultured with exogenous FGF10, indicating that they can be regulated by signaling pathways common to FGFRs and Ret. Studies with signaling inhibitors suggested that PI3K (activated by both Ret and FGFRs) is one such pathway. The inability of FGF7 to upregulate *Etv4*/*Etv5* may underlie the distinct effects of FGF7 and FGF10 on branching of isolated UBs; only the latter induces formation of elongated tubules with distinct ampullae 46.

Etv4 and *Etv5* are most strongly expressed in the WD/UB lineage, and in the *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ mutants, the UB either failed to emerge from the WD or was defective in later branching. This is consistent with a cell-autonomous role of *Etv4/Etv5* within UB cells, downstream of

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GDNF/Ret signaling. It is possible that *Etv4* and *Etv5* also function in the MM lineage, indirectly, to promote UB growth and branching. However, the normal expression levels of several important mesenchymally-expressed genes (including *Gdnf*) in $Etv4^{-/-};Etv5^{+/-}$ mutant kidneys, as well as the failure of isolated UBs to branch in culture, suggest that the branching defects are intrinsic to the mutant UB epithelium. $Etv4^{-/-};Etv5^{+/-}$ kidneys contained apparently normal glomeruli (in reduced numbers), while the more severely affected $Etv4^{-/-};Etv5^{M/M}$ kidneys lacked any sign of nephrogenesis, which could be due either to a role of *Etv4/Etv5* in the nephron lineage, or to a failure of the mutant UB to induce nephrogenesis.

To begin to identify both direct and indirect targets of *Etv4/Etv5*, we examined the expression of a variety of candidate genes in hypoplastic $Etv4^{-/-};Etv5^{+/-}$ kidneys. Many genes, several of which are regulated by GDNF in the UB tips, showed essentially normal expression. Thus, either a single *Etv5* allele is sufficient for their normal expression, or they are regulated by *Etv4/Etv5*-independent mechanisms (Supplementary Fig. 5). In either case, they are not among the genes responsible for the phenotypic defects in $Etv4^{-/-};Etv5^{+/-}$ kidneys. However, we identified four genes whose expression in the UB tips was strongly reduced in themutants and may contribute to the renal phenotypes.

CXCR4 and its ligand CXCL12 play important roles in cell migration in many situations47. During kidney development, *Cxcr4* is expressed similarly to *Etv4/Etv5*, in the WD and UB tips, MM and nascent nephrons (Fig. 6, data not shown). In our microarray screen, *Cxcr4* was highly upregulated by GDNF, and its expression was barely detectable in either *Ret*-hypomorphic or $Etv4^{-/-};Etv5^{+/-}$ kidneys. In a breast cancer cell line, *Cxcr4* is regulated by the ETS factor Ets148, which binds the same GGAA/T core motif as Etv4 and Etv5, suggesting that *Cxcr4* may be a direct Etv4/Etv5 target in UB cells. CXCR4 might contribute to *Ret*-dependent epithelial cell movements during renal branching morphogenesis49,50, although the *Cxcr4* knockout *alone* has no major effects on UB branching51.

Myb encodes a transcription factor with documented roles in hematopoiesis, colon crypt maintenance, adult brain neurogenesis, and cancer52. Interestingly, in these organs it seems to promote the proliferation of a stem or progenitor cell population, while dampening cell differentiation52. Given its specific expression in UB tips, a proliferative cell population21 that both self-renews and gives rise to differentiated derivatives in the UB trunks49,53 (P. Riccio and F.C., unpublished data), *Myb* might function downstream of *Ret* and *Etv4/Etv5* to maintain the proliferation of tip cells. Transcription of *Myb*, like *Cxcr4*, can be regulated by Ets154,55, and thus it might be a direct Etv4/Etv5 target in kidney. While kidney defects were not reported in *Myb*−/− mice, which die at E15.5, or in hypomorphic *Myb* mutants that survive postnatally, this might be due to redundancy with the closely related gene *Myb*l252 which is also expressed in the UB (unpublished data).

We examined *Met* because it is upregulated by *Gdnf* and *Etv4* in motor neurons39, and is a direct Ets1 target in cell lines 56. Its expression in UB tips was greatly reduced in both $Etv4^{-/-};Etv5^{+/-}$ and *Ret*-hypomorphic kidneys. HGF, the Met ligand, is expressed in the MM and stroma57, and promotes branching in MDCK cell and renal organ cultures57,58.

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Furthermore, a UB-specific knockout revealed that *Met* contributes to UB branching in cooperation with *Egfr*35. In motor neurons, *Gdnf* first induces *Etv4* expression, which activates *Met* expression, allowing HGF to further induce *Etv4*39. A similar positive feedback mechanism might occur in the kidney (Supplementary Fig. 5).

Matrix metalloproteases (MMPs) promote branching morphogenesis by degrading components of the extracellular matrix (ECM), thus remodeling the ECM and releasing bound growth factors41,42. Several *Mmp* genes have been identified as potentially direct Etv4 targets59, and among these, *Mmp14* is expressed in the UB37,43 and can be transactivated by Etv440. MMP14 has been implicated in branching in kidney36 and UB cell cultures37, and *Mmp14* −/− mice have hypoplastic kidneys, suggesting a branching defect38 (R. Zent and K. Riggins, personal communication). We found that MMP14 expression was considerably reduced in both $Etv4^{-/-}$;$Etv5^{+/-}$ and *Ret*-hypomorphic kidneys, indicating that another function of *Etv4/Etv5* is to upregulate *Mmp14* in the UB.

In conclusion, our studies shows that Etv4 and Etv5 are key components of a gene network downstream of *Ret* that promotes and controls renal branching morphogenesis. As *RET* mutations in humans result in renal abnormalities8, these findings are likely to be important for our understanding of the causes of renal birth defects in humans.

Methods

Ureteric bud cultures and Affymetrix array analysis

Ureteric buds were dissected from Swiss Webster mouse embryos at E11.5 and cultured in Matrigel (BD Biosciences) with 2X-concentrated conditioned medium from BSN cells, 10% fetal calf serum, with or without 100 ng/ml GDNF46. They were then removed from the Matrigel, homogenized in TRIzol plus 25 ng/μl glycogen, and stored at −80°C. RNA was isolated from batches of 30–40 pooled UBs, and quantified and checked for integrity using the Agilent 2100 Bioanalyzer. Approximately 8 μg total RNA (from ~200–250 UBs) was used to generate an amount of unamplified probe sufficient for each Affymetrix array. The RNAs were subjected to reverse transcription, second-strand synthesis, and probe generation according to the Expression Analysis Technical Manual (Affymetrix). The same five probe preparations (from three pools of UBs cultured +GDNF and two pools −GDNF) were used for hybridization to U74Av2 and 430A arrays. Hybridization, scanning and low level analysis (generation of raw expression data) were performed by a core facility of the Herbert Irving Comprehensive Cancer Center. Sample comparisons and statistical analysis were performed with dChip 1.3 60, using the Comparison Analysis feature to identify genes differentially expressed between the two groups. The following filtering criteria were used: the "lower limit" for fold-change between the means of the +GDNF and −GDNF samples must exceed 1.2, with 90% confidence limit; and the absolute difference between the two group means must exceed 100.

Mouse strains

$Etv4^{tm1Arbr}$(also called *Etv4-lacZ)*10, $Etv5^{tm1Kmm}$ 11, *Fgf10* 61, *Fgfr2* UB-specific knockout29, $Ret^{tm2(RET)Vpa}$ 15, Ret^{Y1062F} 31 and Ret^{Y1015F} mice 30 have been described. To

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generate the *Etv5-lacZ* targeting vector, a partial genomic clone of *Etv5* was isolated from a strain129/Sv mouse genomic phage library. A 4Kb PstI-XhoI DNA fragment containing exon 10 and part of exon 11 was cloned 5′ to IRES::lacZ and PGK::neo in pBluescript. A 5.2Kb BamHI-NotI *Etv5* genomic fragment containing exon 13 was used for the 3′ homology arm (NotI is from phage sequence). The vector was electroporated into W9.5 ES cells and targeted clones identified by Southern blotting (not shown). The targeted allele thus lacks most of exon 11 and all of exon 12, resulting in loss of the DNA binding domain. Structure of the $Etv4^{lacZ}$ and $Etv5^{lacZ}$ alleles and genotyping is described in Supplementary Fig. 6. $Etv5^{tm1Kmm}$ animals were genotyped by PCR as described11.

Immunofluorescence

Cryosections were washed in PBS, permeabilized in PBS/0.1% Triton, and blocked in blocking buffer (PBS, 0.05% Triton-X 100, and 1% normal donkey serum). Antibodies against Etv4 (1:50)62 or MMP14 (1:200, Sigma) were diluted in blocking buffer. Cy2- or Cy3- conjugated secondary antibodies (Jackson ImmunoResearch) were diluted in PBT. Images were taken with Zeiss LSM510 Meta or Axio Observer microscopes.

In situ hybridization

Embryos or dissected kidneys were fixed in 4% paraformaldehyde overnight at 4°C and cryosections were obtained by standard procedures. For vibratome sections, fixed tissues were washed in PBS and embedded in 0.3% gelatin/20% BSA/13% sucrose, which was polymerized with 1% glutaraldehyde. 75μm sections were cut and fixed for 20 min. in 4% paraformaldehyde before whole-mount ISH, which was carried out using digoxigenin-labeled probes as described63.

Organ cultures and induction with growth factor-soaked beads

Kidneys or lungs were isolated at the indicated stages and cultured on Transwell filters (Fisher) at the air-medium interface in DMEM with 10% fetal calf serum, 1% glutamine, 1% penicillin/streptomycin at 37°C and 5% CO_2. For GDNF beads, Affigel blue beads (100–200 mesh, Bio-Rad) were washed with PBS/0.1% BSA before incubating with 50ng/μl recombinant GDNF. FGF7 or FGF10 beads were prepared as described64. Anti-GDNF blocking antibody (R&D Systems) was used at 5 μg/ml.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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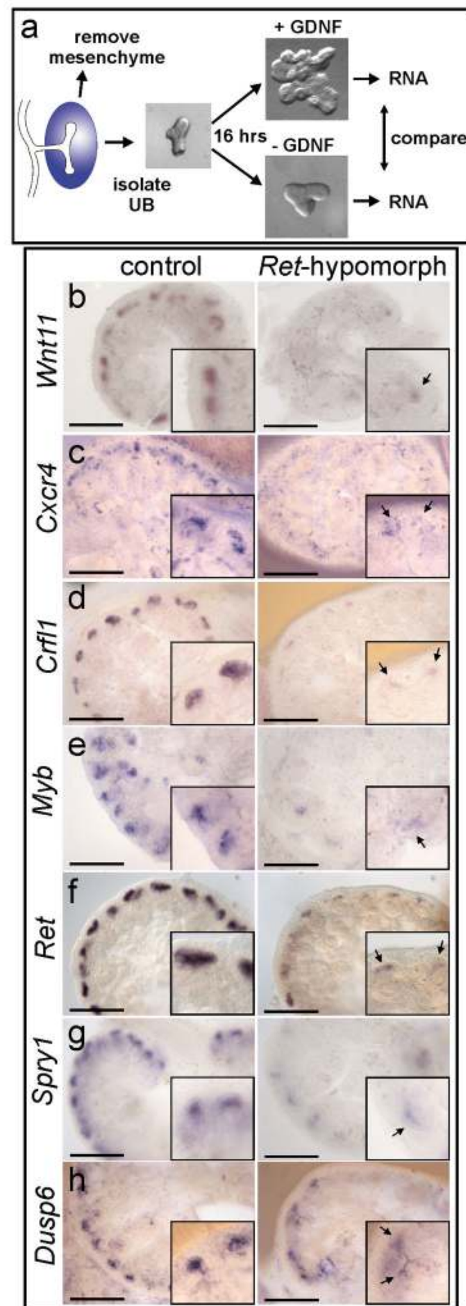

Figure 1. A screen for genes upregulated by GDNF/Ret signaling in the ureteric bud

a, E11.5 UBs isolated from mesenchyme were cultured with or without GDNF. RNA was analyzed with Affymetrix U74Av2 and 430A arrays (Table 1 and Supplementary Tables 1 and 2). **b–h**, *in situ* hybridization to control or $Ret^{tm2(RET)Vpa}/Ret^{tm2(RET)Vpa}$ kidneys (E14.5–E15.5). Unlike *Ret*−/− mice, which usually have renal agenesis, $Ret^{tm2(RET)Vpa}$ homozygotes (expressing only the Ret51 isoform) have a milder phenotype with reduced branching. Thus, the $Ret^{tm2(RET)Vpa}$ allele behaves as a hypomorph due to decreased Ret signaling activity15. Scale bars 100 μM. Insets, 2x enlargements of UB tips (arrows).

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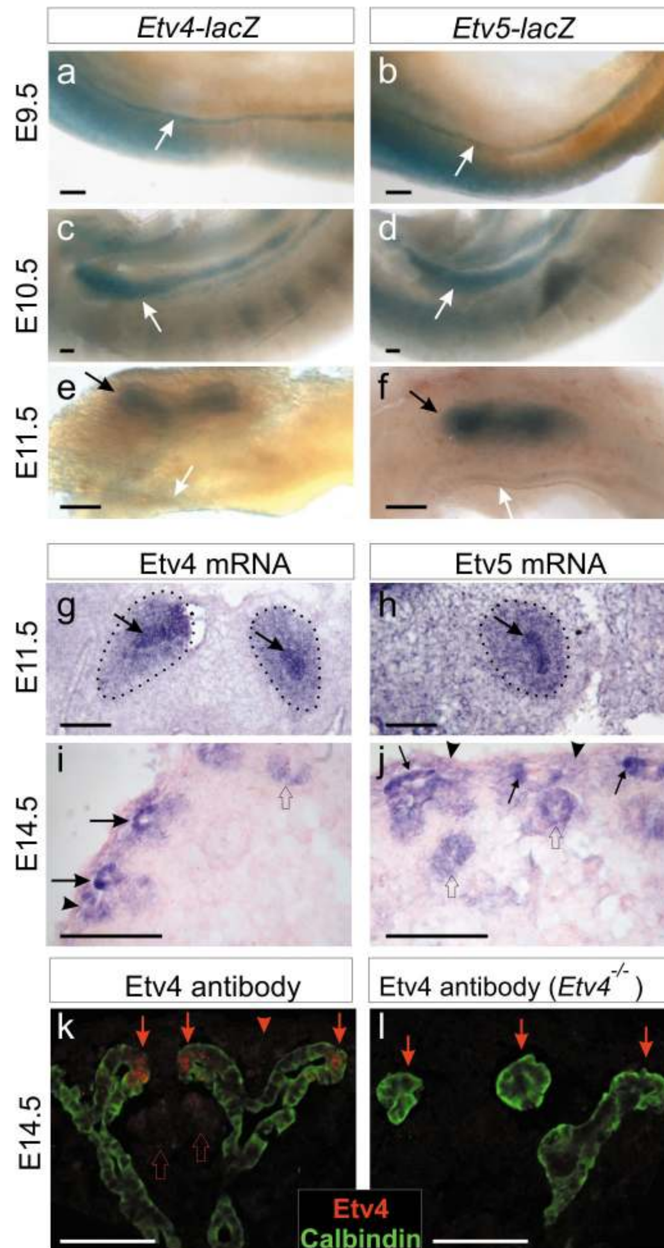

Figure 2. Similar expression patterns of *Etv4* and *Etv5* during kidney development

a–f, expression of *Etv4-lacZ* and *Etv5-lacZ* at E9.5–11.5 in WD (white arrows) and UB (black arrows). **g–h,** *Etv4* and *Etv5* ISH at E11.5. Arrows, UB; dotted lines delineate MM. **i–j**, *Etv4* and *Etv5* ISH in E14.5 kidneys. Arrows, UB tips; arrowheads, MM; open arrows, nascent nephrons. **k**, Etv4 protein expression (red) in UB tips (arrows), MM (arrowheads) and nascent nephrons (open arrows); **l**, lack of Etv4 staining in $Etv4^{-/-}$ kidney shows antibody specificity. Scale bars 100 μM except for k,l (50 μM).

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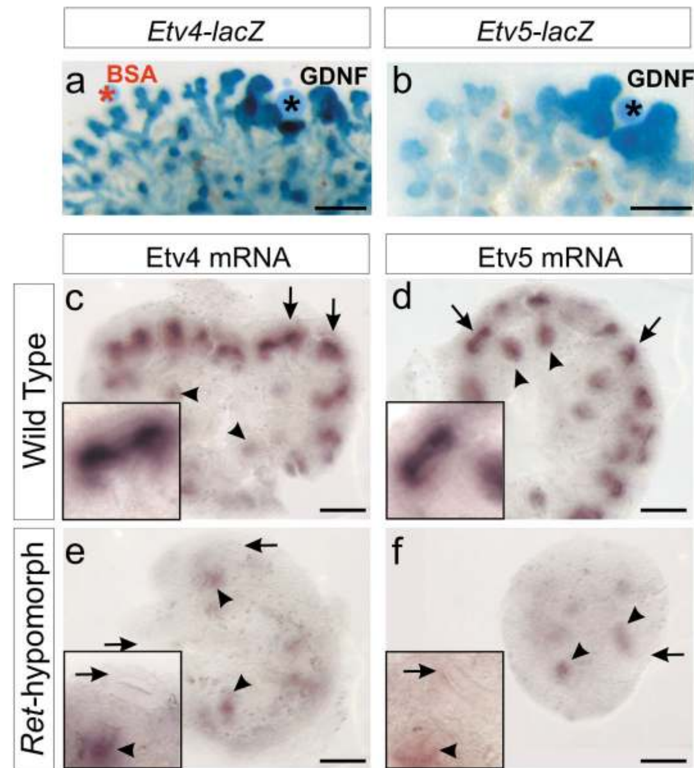

Figure 3. GDNF/Ret signaling regulates *Etv4* and *Etv5* expression in UB tips

a–b, Increased expression of *Etv4-lacZ* and *Etv5-lacZ* in the vicinity of GDNF-soaked beads (black asterisks) but not control BSA-soaked beads (red asterisk). E15.5 (a) or E13.5 kidneys (b) were cultured for 48 hrs. **c–f**, Greatly reduced expression of Etv4 and Etv5 mRNAs in UB tips (arrows) of *Ret*-hypomorphic E14.5 kidneys (e, f) compared to WT kidneys (c, d). However, expression in nascent nephrons (arrowheads) persists in the mutant, consistent with the lack of *Ret* expression in the developing nephrons. Insets in c–f, enlargement of UB tips. Scale bars 100 μM.

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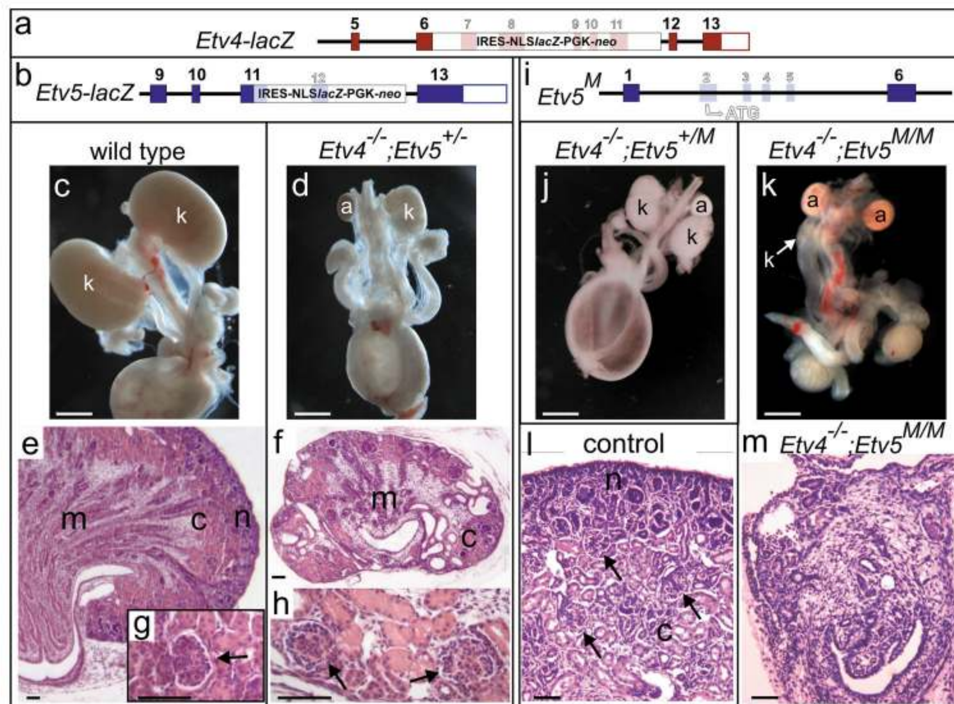

Figure 4. Severe renal developmental defects in compound *Etv4/Etv5* mutant newborn mice with two different *Etv5* knockout alleles

a–b, *Etv4-lacZ* and *Etv5-lacZ* alleles. c–h, renal defects in $Etv4^{-/-};Etv5^{+/-}$ compound mutants. c, normal kidneys (k) in WT. d, unilateral renal agenesis and unilateral hypodysplasia in $Etv4^{-/-};Etv5^{+/-}$ mutant. e–f, histology of WT and $Etv4^{-/-};Etv5^{+/-}$ kidneys. m, medulla; c, cortex; n, nephrogenic zone. **g–h**, enlargements of glomeruli. Note the small size, absent nephrogenic zone, sparse but apparently normal glomeruli, and multiple cysts in mutant kidney. **i**, $Etv5^{M}$ allele. Exons 2–5, including initiation codon, are deleted. **j–m**, renal defects in compound mutants with $Etv5^{M}$ allele. **j**, $Etv4^{-/-};Etv5^{M/+}$ mutant with bilateral hypoplasia. **k**, $Etv4^{-/-};Etv5^{M/M}$ double homozygote with one ureter and tiny kidney rudiment. **l**, histology of normal kidney, with numerous glomeruli (arrows). **m**, histology of the $Etv4^{-/-};Etv5^{M/M}$ kidney rudiment in k, with few epithelial elements and no glomeruli. Scale bars:1 mm in c, d, j, k; 25 μM in other panels.

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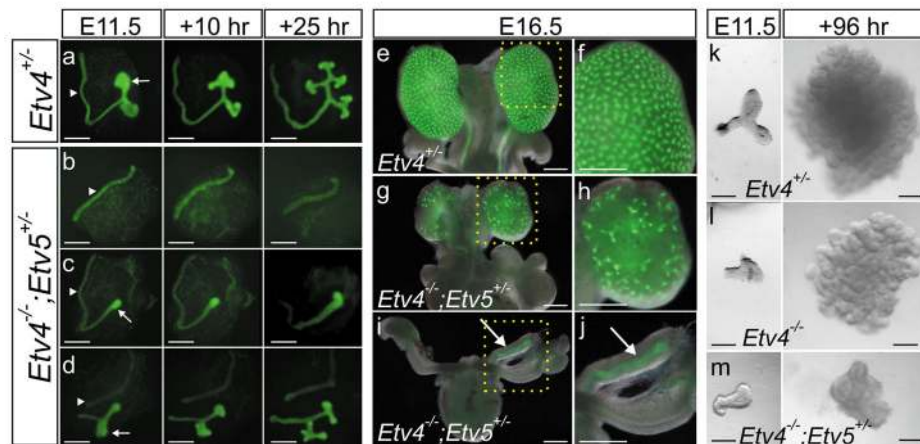

Figure 5. Ureteric bud branching defects in *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ compoundmutants

a–d, culture of one control (a) and three representative compound mutant E11.5 kidney primordia, all carrying *Hoxb7/myrVenus*. Mutant b has a WD (arrowhead) but fails to make a UB, c has a UB (arrow) that fails to branch, d has a normal T-shaped UB at E11.5 but branches less than control when cultured. **e–j**, *Hoxb7/myrVenus* fluorescence reveals normal UB branching in a control kidney at E16.5 (e–f), reduced and irregular branching in the hypoplastic kidneys of one mutant (g–h), and a single unbranched ureter with no kidney in another mutant (i–j). Boxed areas are enlarged in f, h, j. **k–m**, culture of isolated, mesenchyme-free UBs in Matrigel. The *Etv4*$^{+/-}$ (k) and *Etv4*$^{-/-}$ (l) UBs grow and branch extensively but the *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ (m) does so only slightly. Scale bars 100 μM (a–d, k–m), 500 μM (e–j).

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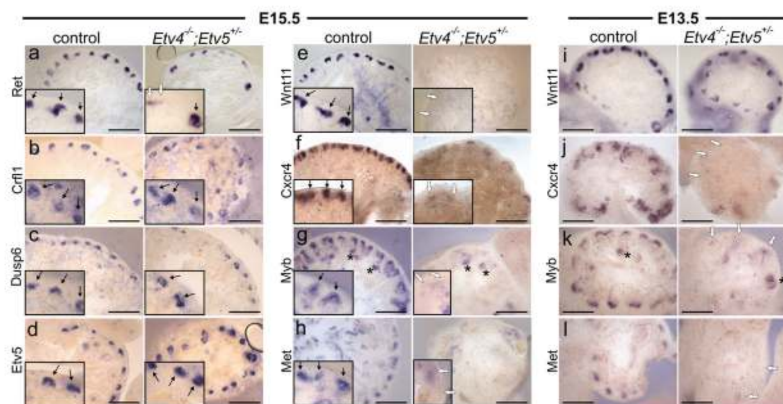

Figure 6. Gene expression in UB tips of *Etv4$^{-/-}$;Etv5$^{+/-}$* compoundmutant kidneys

a–d, Expression of Ret, Crlf, Dusp6 and Etv5 mRNAs was similar in the UB tips of mutant and control kidneys at E15.5, although Ret was reduced in some tips. **e–h**, At E15.5, expression of Wnt11, Cxcr4, Myb and Met was greatly reduced in mutant kidneys. **i–l**, At E13.5, Wnt11 expression was normal, but Cxcr4, Myb and Met were greatly reduced. Insets show 2x enlargements of UB tips. Black arrows indicate tips with normal expression, white arrows those with reduced expression, and asterisks indicate nascent nephrons. Controls include WT, *Etv4$^{+/-}$* or *Etv5$^{+/-}$*. Scale bars 100 μM.

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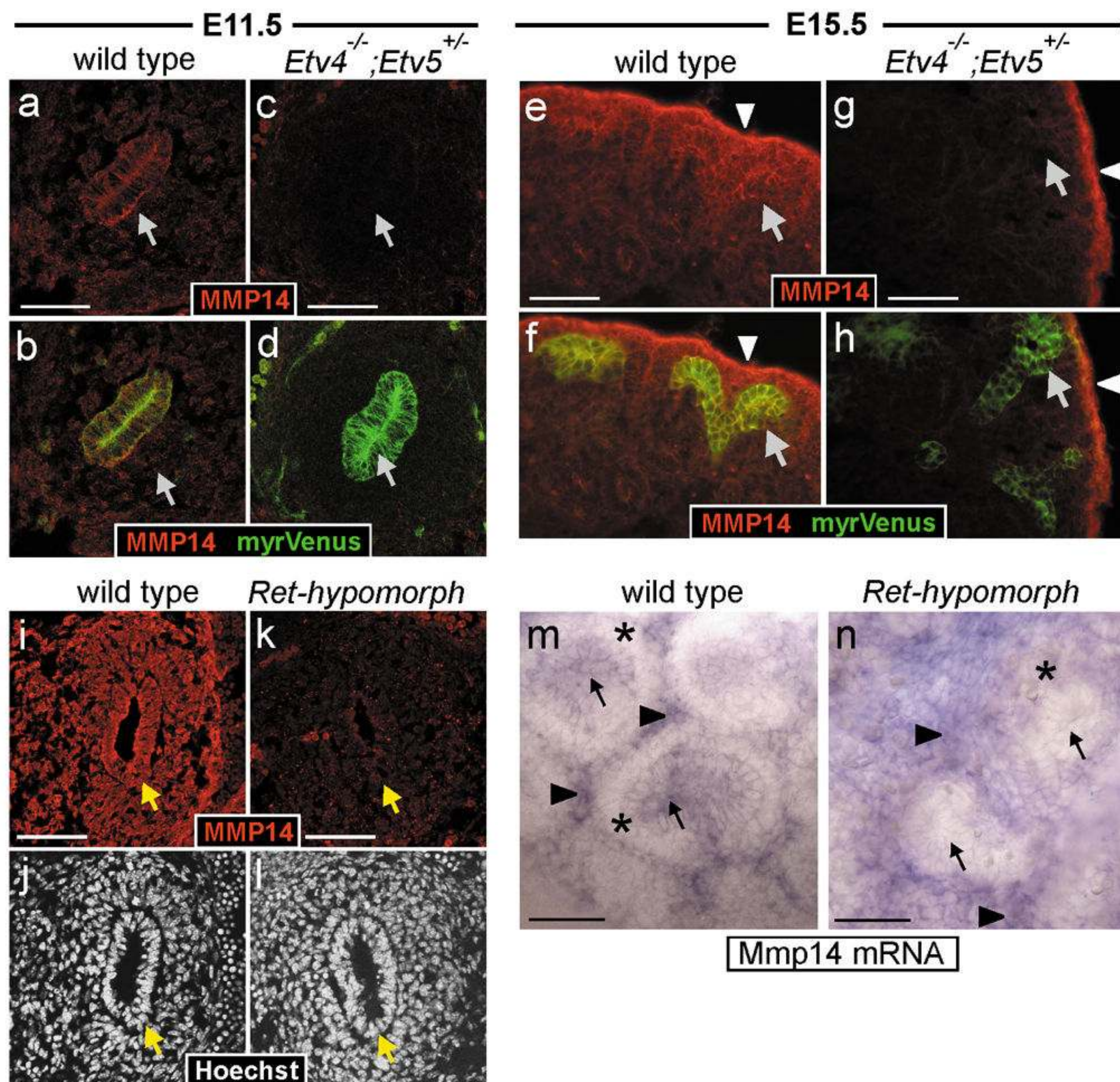

Figure 7. Reduced MMP14 expression in *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ compoundmutant and *Ret*-hypomorphic kidneys

a–h, MMP14 protein in WT and *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ kidneys. At both E11.5 and E15.5, MMP14 is detected in UB epithelium (arrows) and surrounding mesenchyme of WT kidneys (a–b, e–f) but is greatly reduced in UB and MM of *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ kidneys (c–d, g–h). The UB is marked by *Hoxb7/myrVenus* in b, d, f, h. In the *Etv4*$^{-/-}$*;Etv5*$^{+/-}$ at E15.5 (g–h), strong MMP14 expression persists in the peripheral stroma (arrowheads). **i–n**, analysis of *Ret*-hypomorphic kidneys. MMP14 protein (i,k) is reduced in the UB epithelium and the surrounding MM of *Ret*-hypomorphic kidneys at E11.5. In WT E15.5 kidney (m), Mmp14 mRNA is detected in UB epithelium (arrows) and stroma (arrowheads) but not in cap mesenchyme surrounding UB tips (asterisks). In *Ret*-hypomorphic kidney (n), Mmp14

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mRNA is reduced in UB epithelium but persists in stroma (cap mesenchyme is reduced in quantity in the mutant). Scale bars 50 μM.

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Table 1

Genes showing the most elevated expression in UBs cultured with GDNF, including those with an average fold-change of >2.5 in the U74Av2 and 430A arrays, or >3 if represented on only one array (430A * or U74Av2 **).

Gene symbol	**fold-change +/− GDNF**
Wnt11	15
Cxcr4	10
Etv5	5.5*
Crlf1	4.7
Myb	3.8
Arg2	3.5
Car2	3.5**
E2f8	3.3**
Ret	3.3
Spry1	3.3*
Mtm1	3.1
Etv4	2.9
Dusp6	2.9
Ccnd1	2.9
Ncaph	2.7
Ect2	2.5
Spred2	2.6
Ccnb1	2.6
Snrpd3	2.5