

# SLIT2-Mediated ROBO2 Signaling Restricts Kidney Induction to a Single Site

## Short Article

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### Summary

Kidney development occurs in a stereotypic position along the body axis. It begins when a single ureteric bud emerges from the nephric duct in response to GDNF secreted by the adjacent nephrogenic mesenchyme. Posterior restriction of *Gdnf* expression is considered critical for correct positioning of ureteric bud development. Here we show that mouse mutants lacking either SLIT2 or its receptor ROBO2, molecules known primarily for their function in axon guidance and cell migration, develop supernumerary ureteric buds that remain inappropriately connected to the nephric duct, and that the SLIT2/ROBO2 signal is transduced in the nephrogenic mesenchyme. Furthermore, we show that *Gdnf* expression is inappropriately maintained in anterior nephrogenic mesenchyme in these mutants. Thus our data identify an intercellular signaling system that restricts, directly or indirectly, the extent of the *Gdnf* expression domain, thereby precisely positioning the site of kidney induction.

### Introduction

During embryogenesis, the site at which a particular organ forms is determined by localized induction of a subset of the cells that are competent to form that organ. For example, the kidney (metanephros) develops from a subset of nephrogenic cells in the intermediate mesoderm that have been exposed to an inductive signal from a structure known as the ureteric bud (UB) (Saxen, 1987). Thus the site at which the kidney develops depends on where the UB forms.

The UB is an outgrowth of the nephric (Wolffian) duct, which develops lateral to the nephrogenic mesenchyme

(see Figure 1E). In mice, the nephric duct begins to form from anterior intermediate mesoderm at ~embryonic day (E) 8.75 (Bouchard et al., 2002) and subsequently extends caudally until it makes contact at ~E10.5 with the endoderm-derived cloaca, which contains precursors of the bladder and urethra (Kaufman and Bard, 1999). A few hours later, the UB emerges from the duct at a level just rostral to the posterior limit of the hindlimb bud (Saxen, 1987). As it extends into the nephrogenic (metanephric) mesenchyme, the UB branches at its advancing tip, forming a tree-like structure that becomes the collecting duct system of the kidney. The stalk that initially connects the UB to the nephric duct forms the ureter, through which urine drains from kidney to bladder.

Signals from UB branch tips are required for the formation of nephrons, the functional units of the kidney, and for survival of the metanephric mesenchyme (Saxen, 1987; Koseki et al., 1992). Thus, when UB formation fails, the kidney does not develop. Conversely, mutations that cause the formation of a supernumerary UB anterior to the normal one result in the development of an anterior ectopic kidney that fuses with the normal one and of a supernumerary ureter that fails to connect to the bladder (Kume et al., 2000). These observations underscore the critical importance of precisely controlling the process of UB formation for normal kidney development.

UB formation is elicited by GDNF, which is secreted by the metanephric mesenchyme (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) and signals via the receptor tyrosine kinase RET (Pachnis et al., 1993) and its coreceptor GFR $\alpha$ 1 (Vainio and Lin, 2002), which are expressed in nephric duct epithelium. Absence of the ligand or either coreceptor leads to failure of UB formation (Sariola and Saarma, 1999; Dressler, 2002; Vainio and Lin, 2002), demonstrating the central role that GDNF-mediated RET signaling plays in this process. At the time of UB formation, *Ret* and *Gfr $\alpha$ 1* are expressed along the entire anterior-posterior (A-P) extent of the nephric duct, whereas *Gdnf* expression has become restricted to the vicinity of the nascent UB (Pachnis et al., 1993, and our unpublished data). The finding that GDNF-soaked beads can elicit ectopic UB formation in organ culture (Sainio et al., 1997; Brophy et al., 2001) shows that the duct epithelium is competent to respond to GDNF in regions anterior to the normal site of UB formation, and suggests that posterior restriction of *Gdnf* expression is critical for preventing supernumerary UB formation. Consistent with this model, anterior expansion of the *Gdnf* expression domain is correlated with supernumerary UB formation in *Foxc1* mutants (Kume et al., 2000).

Here we demonstrate that another intercellular signaling system, involving SLIT2 and its receptor ROBO2, plays a key role in controlling UB formation. In vertebrates, the three Slit genes encode large secreted proteins that signal via ROBO receptors, and are best known for their function as chemorepellents that cause axons or migrating cells to turn away from the source

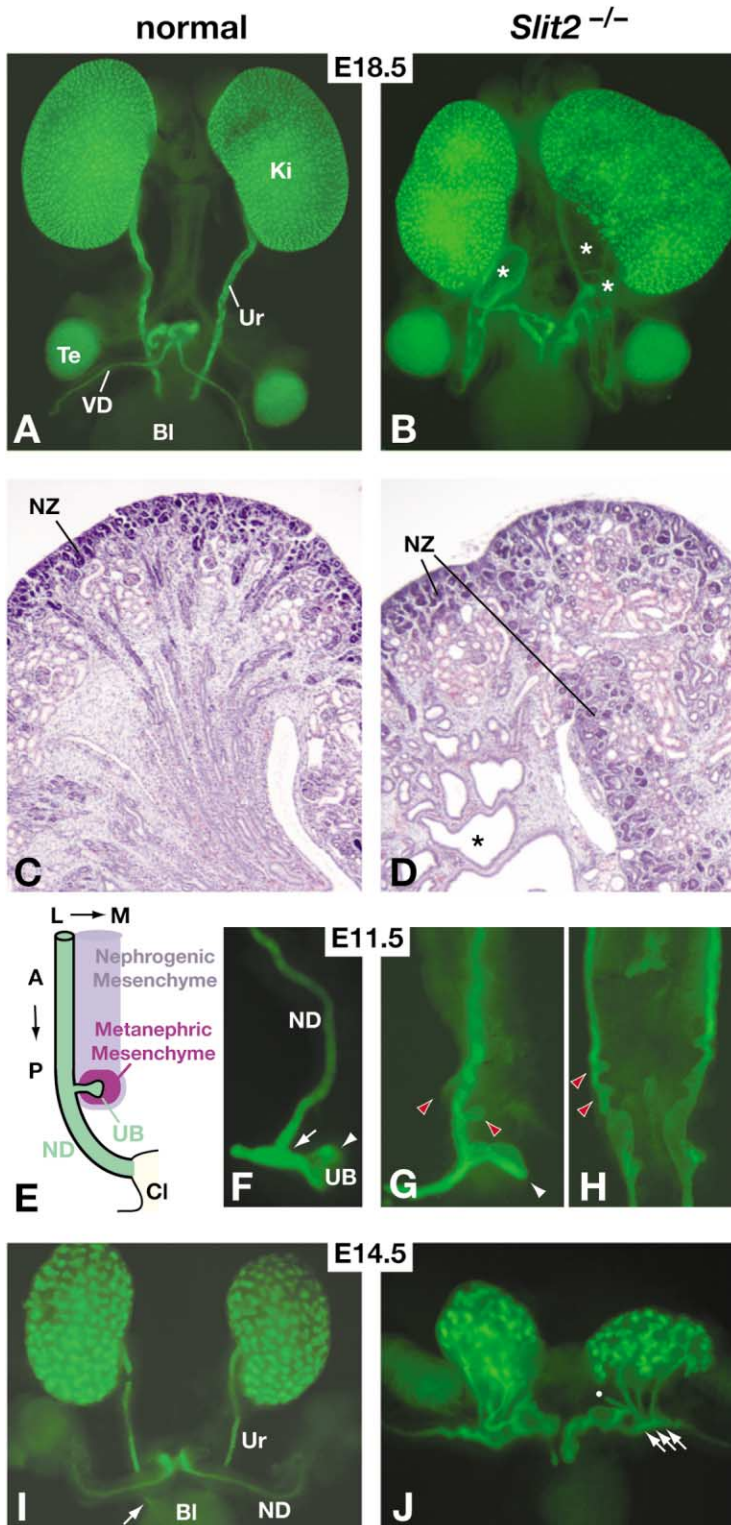
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**Figure 1. Loss of *Slit2* or *Robo2* Function Results in Abnormal Kidney Development Due to Supernumerary Ureteric Bud Formation**

(A and B) Urogenital system of a normal and a *Slit2*<sup>-/-</sup> embryo at E18.5 is shown in whole mount (ventral view). Nephric duct derivatives are visualized by expression of a *Hoxb7*-GFP transgene (Srinivas et al., 1999). Asterisks in (B) are in the lumens of hydroureters. (C and D) Histological sections of E18.5 normal and *Slit2*<sup>-/-</sup> kidneys stained with hematoxylin and eosin. Asterisk in (D) marks a dilated, fluid-filled collecting duct in the mutant kidney. The region where nephrogenesis occurs (nephrogenic zone) is restricted to the periphery in the normal kidney, but extends into the interior of the *Slit2*<sup>-/-</sup> kidney. (E) Schematic diagram illustrating the tissues at ~E11.5 from which the kidney develops (ventral view). The nephric duct forms lateral to the nephrogenic mesenchyme, which gives rise posteriorly to the definitive kidney (metanephros). Although the diagram shows the UB extending toward the midline (medially), it actually extends dorsally, below the plane of the page. (F–H) Analysis at E11.5 shows a single UB in the normal embryo (visualized by *Hoxb7*-GFP expression) and multiple UBs in *Slit2*<sup>-/-</sup> embryos (visualized using an anti-cytokeratin antibody that stains most epithelia). The nephric duct is shown in lateral view (dorsal to the right) in (F) and (G), or in ventral view in (H). White arrowheads point to the normal UB, which cannot be seen in the ventral view. Red arrowheads point to ectopic UBs, which do not all project in the same direction as the normal UB. Arrow in (F) points to the connection of the UB to the nephric duct. (I and J) Analysis of *Hoxb7*-GFP expression at E14.5 (ventral view) shows a single ureter in the normal embryo and multiple ureters in *Slit2*<sup>-/-</sup> mutants. White arrows point to the insertion site of the ureter into the bladder (I), or into the nephric duct (J). White dot in (J) indicates blind-ending ectopic UB. Abbreviations: A, anterior; BI, Bladder; CI, cloaca; Ki, kidney; L, lateral; M, medial; ND, nephric duct; NZ, nephrogenic zone; P, posterior; Te, testis; UB, ureteric bud; Ur, ureter; VD, vas deferens.

of SLIT (Brose and Tessier-Lavigne, 2000; Wong et al., 2002). We report that inactivation of either *Slit2* or *Robo2* in mice leads to supernumerary UB development, and

that this is correlated with abnormal maintenance of *Gdnf* expression in anterior nephrogenic mesenchyme. We discuss possible mechanisms by which SLIT2/

ROBO2 signaling might function in the normal embryo to ensure that a single UB forms at the appropriate location.

## Results

### Abnormal Kidney Development in *Slit2* Mutants Is Due to Formation of Supernumerary Ureteric Buds

In the course of examining *Slit2* null homozygotes (Plump et al., 2002), which almost all die at birth, we detected two major abnormalities in kidney development at E18.5. First, the collecting ducts and ureters were grossly dilated (hydronephroses) (Figures 1A–1D). Second, the region where new nephrons are being generated, which is normally restricted to the periphery of the developing kidney (Figure 1C), extended into the interior of the mutant kidneys (Figure 1D). Other aspects of kidney development, such as nephron and collecting duct formation, appeared normal.

To determine the cause of these abnormalities, we examined mutant embryos shortly after kidney development is initiated. Normally, by E11.5 a single UB has emerged from the nephric duct and invaded the metanephric mesenchyme (Figures 1E and 1F). However, in *Slit2*<sup>-/-</sup> mutants we observed three or more UBs in 14/16 and two in 2/16 nephric ducts examined (Figures 1G and 1H and data not shown). In contrast to normal UBs, which project dorsally (Figure 1F), some of the supernumerary UBs projected medially (Figures 1G and 1H).

By ~E14.5, the single UB on each side of the normal embryo has branched in the metanephric mesenchyme to form the prospective collecting ducts. In E14.5 *Slit2*<sup>-/-</sup> embryos, we found that although some UBs ended blindly, most had extended into the nephrogenic mesenchyme and branched (Figure 1J), thus inducing two or more adjacent kidneys. Fusion of these multiple kidneys may account for the interior nephrogenesis observed in *Slit2*<sup>-/-</sup> kidneys at E18.5 (Figure 1D).

Also by E14.5, the stalk of the normal UB has elongated to form the ureter (Figure 1I) and undergone a remodeling process whereby it is disconnected from the nephric duct (arrow in Figure 1F) and connected to the bladder (arrow in Figure 1I). As expected from the presence of multiple UBs, we found that all *Slit2*<sup>-/-</sup> kidneys had two or more ureters (3/17 *Slit2*<sup>-/-</sup> kidneys had two, 14/17 had more). Significantly, all of the ureters in *Slit2* mutant embryos remained connected to the nephric duct (Figure 1J), thus precluding normal drainage of urine to the bladder and accounting for the hydronephroses observed in older mutant embryos. These data indicate that SLIT2 plays an essential role in normal kidney development, functioning to ensure that a single UB forms and that the ureter connects normally to the bladder.

### *Robo2* Null Homozygotes Have a Kidney Phenotype Similar to that of *Slit2* Mutants

SLITs are known to signal via ROBO receptors, including ROBO1 and ROBO2 in vertebrates (Brose and Tessier-Lavigne, 2000; Wong et al., 2002). Since kidney development is normal in *Robo1* mutant homozygotes (Xian et al., 2001, and our unpublished data), we explored the

possibility that SLIT2 signals via ROBO2 to prevent supernumerary UB formation by producing mice carrying a *Robo2* deletion allele that does not produce any functional *Robo2* RNA (Figures 2A–2C). We confirmed that this allele produces no functional ROBO2 protein by demonstrating a lack of ROBO2 immunoreactivity in the mutant spinal cord (data not shown).

Like *Slit2*<sup>-/-</sup> mice, almost all *Robo2*<sup>-/-</sup> pups failed to survive after birth, but they were present at Mendelian frequency at E15.5–E16.5. Significantly, the *Robo2* mutants exhibited a multiple ureter phenotype similar to, albeit slightly milder than, the one observed in *Slit2* mutants: 6/14 *Robo2*<sup>-/-</sup> kidneys had three ureters, 6/14 had two ureters, and 2/14 had only one (Figure 2D). This reduced severity might be due to differences in genetic background, or functional redundancy with *Robo1*, which is coexpressed at a low level with *Robo2* (Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/6/5/709/DC1]). Another ROBO-related gene, *Rig1/Robo3*, is unlikely to be involved because its expression was not detected in the developing kidney at the time of UB formation (data not shown). Most *Robo2* mutant ureters, like those in *Slit2* mutants, remained connected to the nephric duct (Figure 2D), and in only 2/14 kidneys examined did one of the multiple ureters remodel and insert into the bladder. These data provide genetic evidence that the SLIT2 signal that is essential for suppressing supernumerary UB formation and for ureter remodeling is transduced by the ROBO2 receptor.

### SLIT2/ROBO2 Signaling Functions in the Nephrogenic Mesenchyme to Suppress Supernumerary Ureteric Bud Formation

The data described above indicate that SLIT2/ROBO2 signaling functions anterior to the normal site of UB development to prevent the formation of supernumerary UBs. To gain insight into which tissues produce and transduce the SLIT2/ROBO2 signal, we performed an in situ hybridization analysis of *Slit2* and *Robo2* expression in near-adjacent transverse sections taken at three different A-P levels through the hindlimb region of normal ~E10.5 embryos, a few hours before the UB emerges from the nephric duct at the 38–39 somite stage (n = 2; Figures 3A–3G). We also assayed for *Gdnf* expression, which marks nephrogenic mesenchyme (Figures 3H–3J). *Slit2* RNA was detected at a high level throughout the nephric duct and at a relatively low level in the nephrogenic mesenchyme (Figures 3B–3D). Interestingly, expression in the nephrogenic mesenchyme appeared to be significantly higher anteriorly (Figure 3B), where *Gdnf* expression was not detected (Figure 3H).

In contrast, *Robo2* RNA was detected at a high level in the nephrogenic mesenchyme throughout the region examined (Figures 3E–3G). It was also detected at a lower level in the nephric duct, but only in sections through the region where the UB will emerge, as well as immediately anterior to it (Figure 3G, and data not shown). Thus, in the anterior region where SLIT2/ROBO2 signaling functions to suppress the formation of supernumerary UBs, *Robo2* expression appears to be restricted to the nephrogenic mesenchyme, indicating that this is the tissue in which the SLIT2 signal is transduced.

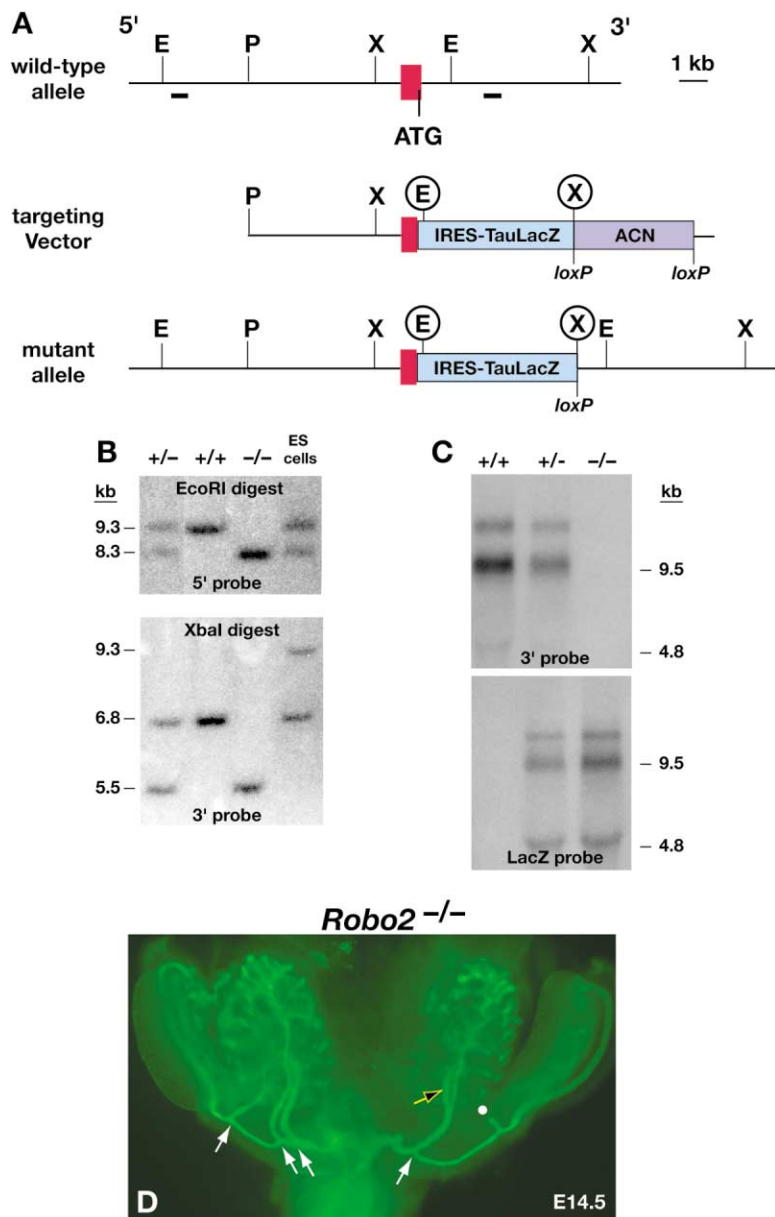


Figure 2. Loss of *Robo2* Function Results in Development of Multiple Ureters

(A) Schematic diagram of the *Robo2* gene (upper illustration) and the targeting vector (middle illustration) used to produce a *Robo2* deletion allele. In the targeting vector, 135 bp of *Robo2* DNA, including the 3' end of the putative first exon (red box), which contains the ATG translational start codon, and the 5' end of the adjacent intron, was replaced with an IRES-tauLacZ expression cassette (Mombaerts et al., 1996) and ACN, a self-excising floxed sperm-specific cre recombinase/neomycin-resistance expression cassette (Bunting et al., 1999). When the mutant allele is transmitted through the male germline, expression of cre results in the deletion of the floxed cre/neo cassette (lower illustration).

(B) Genotype analysis of mice carrying the *Robo2* mutant allele. Southern blots were performed on tail and ES cell DNA digested with EcoRI or XbaI, and hybridized with the probes illustrated in (A) (horizontal bars in upper illustration). The band representing the mutant allele is larger in DNA from ES cells than from mice, because the ACN fragment was deleted when the allele was passed through the germline.

(C) Northern blots were performed on RNA isolated from E14.5 brain and hybridized with the probes indicated.

(D) Visualization of nephric duct derivatives in a *Robo2*<sup>-/-</sup> embryo using an anti-cytokeratin antibody (ventral view). White arrows point to insertion sites of ureters into the nephric duct. White dot indicates a blind-ending ectopic UB. Yellow/black arrow points to a split ureter. Abbreviations: E, EcoRI; P, PflmI; X, XbaI. Circled E and X sites are present only in the inserted DNA.

### *Gdnf* Expression in *Slit2* and *Robo2* Mutants Is Abnormally Maintained in the Anterior Nephrogenic Mesenchyme

Our results imply that the nephrogenic mesenchyme is the tissue affected by loss of SLIT2/ROBO2 signaling; it is also the source of GDNF, which has been shown to be sufficient to induce supernumerary UBs (Sainio et al., 1997; Brophy et al., 2001). This suggested that the supernumerary UB phenotype caused by loss of *Slit2* or *Robo2* function might be due to effects on *Gdnf* expression. To test this possibility, we first performed an analysis in normal nephrogenic mesenchyme of *Gdnf* expression, which is known to be dynamic (Kume et al., 2000) but has not previously been documented in detail. At ~E9.5 (24–28 somites), we detected *Gdnf* RNA in the nephrogenic mesenchyme along most of the A-P length of the body axis (n = 5/6; Figure 4A). During the next 2

days of development, *Gdnf* expression became progressively restricted to the posterior region where the UB forms. By ~E10.5–E10.75 (35–39 somites), the rostral limit of the *Gdnf* expression domain was almost always detected at the level of the mid-hindlimb bud (Figure 4B); in only 2/14 cases was it detected anterior to that level. After the 40 somite stage (E11.0), it was never detected anterior to that level (n = 25). In most cases, *Gdnf* expression became even more restricted by the 44–45 somite stages, to the immediate vicinity of the developing UB (n = 6/8; Figure 4C).

In contrast, in both *Slit2* and *Robo2* mutant embryos, the *Gdnf* expression domain almost always extended rostral to the level of the mid-hindlimb bud. Thus, in 4/5 *Slit2*<sup>-/-</sup> and 2/2 *Robo2*<sup>-/-</sup> embryos at the 40–44 somite stages, the rostral limit of the *Gdnf* expression domain in the nephrogenic mesenchyme was near the anterior

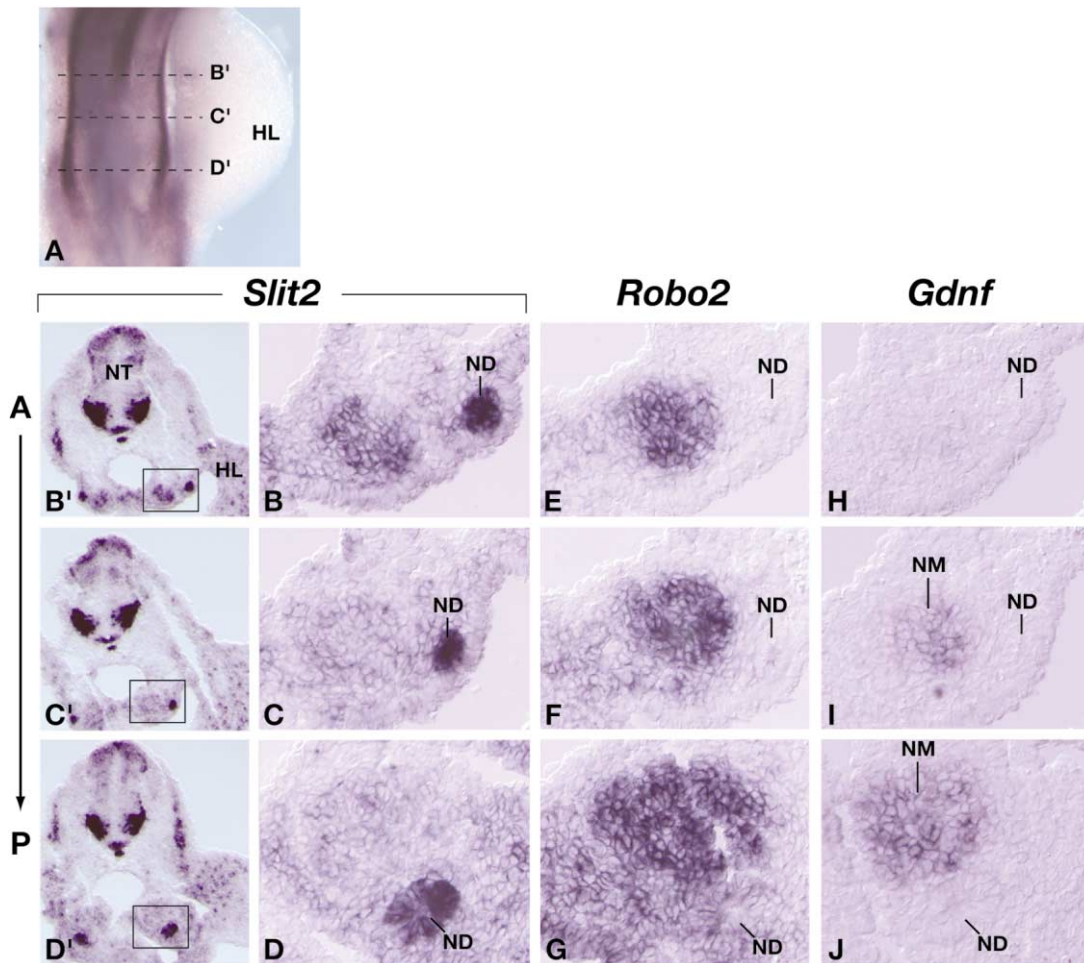


Figure 3. Normal Domains of *Slit2*, *Robo2*, and *Gdnf* Expression at a Stage Just prior to the Emergence of the Ureteric Bud from the Nephric Duct (A) Whole-mount RNA in situ hybridization analysis of *Slit2* in a normal embryo at the 38 somite stage (E10.75; ventral view). (B'–D' and B–J) RNA in situ hybridization analysis of transverse sections through a single E10.5 embryo. (B'–D') Low magnification view of sections taken at different levels along the A–P axis and hybridized with a *Slit2* probe. Boxes indicate the regions shown at higher magnification in (B)–(D). (E)–(J) Near-adjacent sections hybridized with the probes indicated. The approximate A–P levels of the sections in (B')–(D') are indicated by the dashed lines in (A). Abbreviations: HL, hindlimb bud; ND, nephric duct, NM, nephrogenic mesenchyme; NT, neural tube.

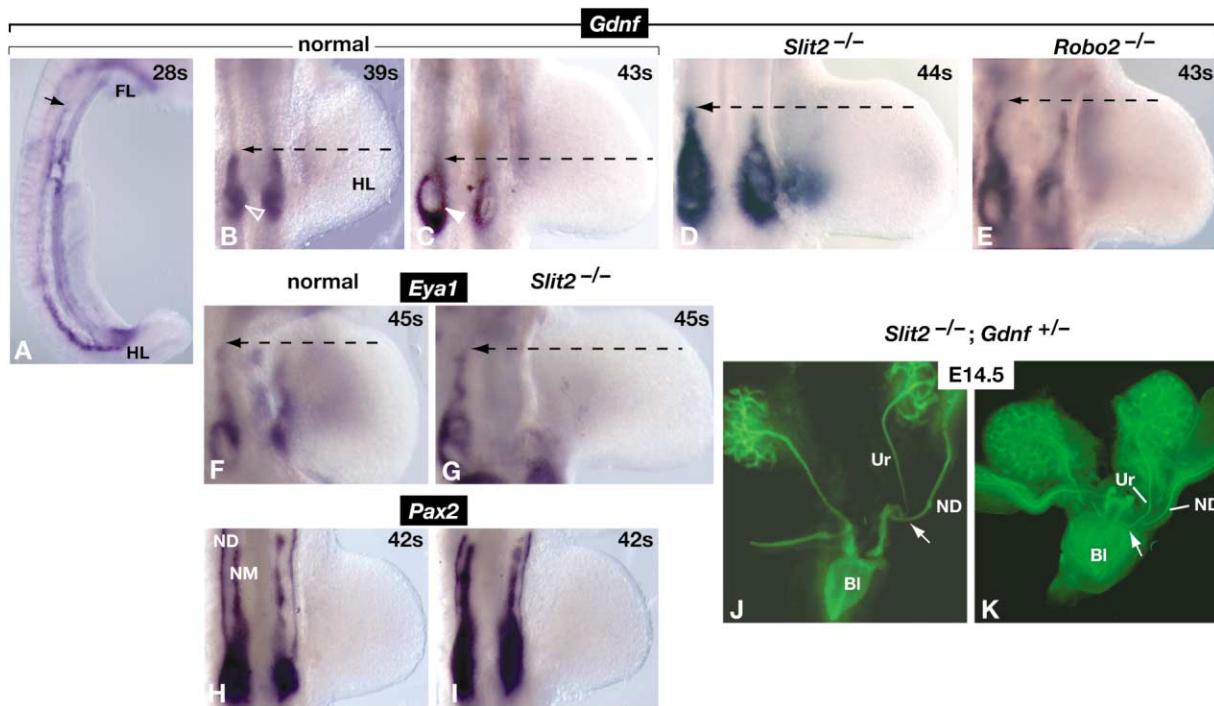
edge of the hindlimb bud (Figures 4D and 4E). These data indicate that in the absence of SLIT2/ROBO2 signaling, *Gdnf* expression is abnormally maintained in the nephrogenic mesenchyme anterior to the site of UB formation, and suggest that in the normal embryo, SLIT2/ROBO2 signaling is required, directly or indirectly, for posterior localization of *Gdnf* expression to the vicinity of the nascent UB.

One possible mechanism by which SLIT2/ROBO2 signaling might perform this function is by repressing transcriptional activation or by stimulating transcriptional repression of *Gdnf*. Candidates for positive transcriptional regulators include EYA1 (Xu et al., 1999) and PAX2 (Brophy et al., 2001), which are coexpressed with *Gdnf* in the metanephric mesenchyme and are required for its expression. Surprisingly, whole-mount in situ hybridization analysis revealed that in normal embryos, *Eya1* (Figure 4F) and *Pax2* (Figure 4H) RNA was detected in a domain whose rostral limit was substantially anterior to that of the *Gdnf* domain (see Figure 4C), and that the

*Eya1* and *Pax2* expression patterns were very similar in normal and *Slit2*<sup>-/-</sup> embryos (Figures 4F–4I). We also assayed for the expression of FOXC1, a candidate transcriptional repressor of *Gdnf*, which is widely expressed in the intermediate mesoderm (Kume et al., 2000), but found no difference between normal and *Slit2*<sup>-/-</sup> embryos (data not shown). These results suggest that loss of *Slit2* function does not cause ectopic UB formation via an effect on the expression of these transcriptional regulators of *Gdnf*.

#### The *Slit2*<sup>-/-</sup> Kidney Phenotype Can Be Rescued by Reducing *Gdnf* Dosage

The process of UB formation is sensitive to the level of *Gdnf* expression, as demonstrated by the fact that *Gdnf* null heterozygotes frequently lack a kidney due to failure of UB formation (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). We therefore were interested to determine whether reducing *Gdnf* dosage would prevent the formation of supernumerary UBs in *Slit2* mutants.



**Figure 4. Abnormal *Gdnf* Expression in the Nephrogenic Mesenchyme Explains the Supernumerary Ureteric Bud Phenotype of *Slit2* and *Robo2* Null Mutants**

(A–I) Whole-mount RNA in situ hybridization to detect expression of the genes indicated, in normal, *Slit2*<sup>-/-</sup>, or *Robo2*<sup>-/-</sup> embryos at various somite stages (s). (A) Oblique lateral view of a normal embryo at the 28 somite stage (with head, forelimb buds, and developing internal organs removed) ~18–22 hr before UB formation initiates. The positions of the forelimb and hindlimb buds are indicated. (B–I) Ventral views of the posterior region of embryos at the somite stages indicated. Black arrows point to the anterior limit of gene expression in the nephrogenic mesenchyme. Open and filled white arrowheads point to the region where the UB will form or has formed, respectively. *Pax2* expression is detected along the entire A–P extent of the nephrogenic mesenchyme and nephric duct. The right hindlimb bud and internal organs have been removed to facilitate probe penetration; detection of signal is often poor on the side where the hindlimb bud is intact.

(J and K) Visualization of nephric duct derivatives in *Slit2*<sup>-/-</sup>; *Gdnf*<sup>+/-</sup> embryos using anti-cytokeratin antibody. In both examples shown here, each kidney has a single ureter. (J) A mutant in which ureter remodeling has not occurred. The white arrow points to the insertion site of the ureter into the nephric duct. (K) A mutant in which the ureter has undergone remodeling. The white arrow points to the site where the ureter connects to the bladder. Abbreviations as in the legends to Figure 1 and Figure 3, and FL, forelimb bud.

In 24/30 *Slit2*<sup>-/-</sup>; *Gdnf*<sup>+/-</sup> mutant kidneys examined at E14.5, only a single ureter was present (Figures 4J and 4K). In the remaining 6/30 kidneys, there were either two ureters or one ureter and a blind-ending ectopic protrusion (data not shown). These results provide genetic evidence that supernumerary UB formation in *Slit2*<sup>-/-</sup> embryos is dependent on *Gdnf* dosage.

Interestingly, of the 15 *Slit2*<sup>-/-</sup>; *Gdnf*<sup>+/-</sup> kidneys with a single ureter that were analyzed for remodeling, 5 had ureters that remained abnormally connected to the nephric duct (Figure 4J), whereas remodeling was rescued in the remaining 10 (Figure 4K). This observation suggests that the ureter remodeling process is also dependent on GDNF.

## Discussion

Restriction of the *Gdnf* expression domain is thought to be a key step in localizing UB formation to the appropriate site (Lechner and Dressler, 1997; Kume et al., 2000). In this study, we provide genetic evidence that SLIT2-mediated activation of ROBO2 is required to prevent formation of supernumerary UBs. Furthermore, we

identify the nephrogenic mesenchyme as the tissue in which the SLIT2/ROBO2 signal is transduced. We also demonstrate that in both *Slit2* and *Robo2* null homozygotes, *Gdnf* expression is inappropriately maintained in anterior nephrogenic mesenchyme, and that the *Slit2* supernumerary UB phenotype can be rescued by reducing *Gdnf* gene dosage. The simplest interpretation of these findings is that the primary function of SLIT2/ROBO2 signaling during kidney development is to ensure that the *Gdnf* expression domain becomes localized to the region where the UB normally forms, thereby restricting kidney induction to the appropriate site.

Our data leave open the question of where the SLIT2 that is required to suppress supernumerary UBs is produced. The nephric duct, which expresses *Slit2* along its length, might be the source of the signal. But if so, there must be a mechanism that prevents SLIT2 produced in the posterior nephric duct from suppressing formation of the normal UB. One possibility is that the level of *Slit2* expression in the posterior nephric duct is too low to activate ROBO2 in the nearby nephrogenic mesenchyme. *Slit2* expression is also observed in the nephrogenic mesenchyme, in what appears to be a reciprocal relationship with *Gdnf* expression (compare

Figures 3B–3D to 3H–3J). These data suggest a model in which the mesenchyme anterior to the region where the UB forms normally produces sufficient SLIT2 to activate ROBO2 in the nephrogenic mesenchyme, resulting in the complete elimination of *Gdnf* expression, whereas more posteriorly, SLIT2 levels are too low to activate ROBO2 signaling, allowing *Gdnf* expression to be maintained.

#### Similarities and Differences between the Kidney Phenotype in *Foxc1* versus *Slit2* and *Robo2* Mutants

The *Slit2/Robo2* kidney phenotype described here is similar in several respects to that caused by loss of *Foxc1* function (Kume et al., 2000). In both cases, supernumerary UBs develop, resulting in multiple ureter formation, and this is correlated with abnormal maintenance of *Gdnf* expression in the region anterior to the site of normal UB formation. These similarities raise the possibility that SLIT2/ROBO2 signaling and FOXC1 may act in the same molecular pathway. Since FOXC proteins are transcriptional regulators, and *Foxc1* is expressed in the nephrogenic mesenchyme, we explored the possibility that FOXC1 might regulate *Slit2* or *Robo2* expression. However, we found that both genes were still expressed in the intermediate mesoderm of *Foxc1* mutant embryos (data not shown), although we cannot rule out the possibility of subtle changes in their expression domains. These data suggest that FOXC1 may function independent of SLIT2/ROBO2 signaling to suppress supernumerary UB formation or may regulate expression of a gene that is required for SLIT2/ROBO2 signal transduction.

Despite their similarities, *Foxc1* mutants differ in one important respect from *Slit2* and *Robo2* mutants: in *Foxc1* mutant kidneys, one of the multiple ureters undergoes remodeling and inserts properly into the bladder (Kume et al., 2000). This suggests that the complete lack of ureter remodeling in all *Slit2* and most *Robo2* mutants is not a consequence of supernumerary UB formation per se, and that remodeling requires *Slit2* and *Robo2* function. This conclusion is further supported by the observation that when *Gdnf* dosage is reduced in *Slit2* mutants and the supernumerary UB phenotype is rescued, the single ureter that develops in the *Slit2*<sup>-/-</sup>; *Gdnf*<sup>+/-</sup> kidneys fails to remodel and remains connected to the nephric duct in 33% of cases. Surprisingly, remodeling occurs normally in the remaining *Slit2*<sup>-/-</sup>; *Gdnf*<sup>+/-</sup> kidneys with one ureter, suggesting that GDNF signaling also plays a role in that process. This conclusion is consistent with data indicating that the GDNF receptor RET is required for ureter remodeling (Batourina et al., 2002).

#### Possible Mechanisms by which SLIT2/ROBO2 Signaling Suppresses Supernumerary Ureteric Bud Formation via an Effect on the *Gdnf* Expression Domain

Much of the current literature on the function of SLIT/ROBO signaling is focused on its chemorepulsive role in cell motility (Brose and Tessier-Lavigne, 2000; Wong et al., 2002). Therefore it is appealing to consider a model

in which SLIT2/ROBO2 signaling eliminates *Gdnf* expression by providing a chemorepulsive signal to *Gdnf*-expressing cells present in anterior nephrogenic mesenchyme, causing them to move posteriorly and thereby accumulate in the condensing metanephric mesenchyme at the stage when the UB begins to form. To explore this possibility, we explanted intermediate mesoderm from normal embryos, labeled nephrogenic mesenchymal cells in the region anterior to the prospective site of UB formation with a lipophilic dye, and assessed whether they had moved posteriorly toward the vicinity of the nascent UB after 24 hr of culture. However, we never observed significant movement of cells in the nephrogenic mesenchyme (data not shown).

A second possible mechanism by which SLIT2/ROBO2 signaling might function to eliminate *Gdnf*-positive cells from the region anterior to the site of UB formation is by inducing cell death. If that were the case, one would expect that in the normal embryo, dying cells would be present in the nephrogenic mesenchyme immediately anterior to the *Gdnf*-positive domain as it is becoming restricted to the prospective site of UB formation. However, we detected few or no dying cells in this region (data not shown).

Furthermore, we think it is unlikely that loss of anterior *Gdnf* expression is achieved by removal of *Gdnf*-positive cells via an effect on cell migration or survival, because *Robo2*-expressing cells, which are presumably SLIT2 responsive, are present anterior to the *Gdnf*-positive domain in normal E10.5 embryos (compare Figure 3E to 3H). If *Robo2*-positive cells exposed to SLIT2 had migrated posteriorly or had died in order to eliminate *Gdnf* expression, one would not expect to find *Robo2*-expressing cells anterior to the *Gdnf*-positive domain. Instead, it seems plausible that these *Robo2*-positive cells have responded to SLIT2 by turning off *Gdnf* expression.

Therefore we favor a third possible mechanism involving effects on *Gdnf* expression to explain how SLIT2/ROBO2 signaling restricts the size of the *Gdnf*-positive domain. ROBO2 activation may downregulate *Gdnf* expression by negatively affecting transcriptional activators or positively affecting transcriptional repressors of *Gdnf*. To explore this issue, we analyzed the expression of *Eya1*, *Pax2*, and *Foxc1*, presumed transcriptional regulators of *Gdnf*, in *Slit2* mutant embryos, but could find no evidence that loss of *Slit2* function affects their expression (Figures 4F–4I, and data not shown).

One unexpected finding was that in normal embryos *Eya1* and *Pax2* expression is detected not only where *Gdnf* is expressed, but also in the region anterior to the *Gdnf*-positive domain (Figures 4F and 4H). Thus, downregulation of *Gdnf* does not appear to be caused by a loss of *Eya1* or *Pax2* expression, but instead may occur via an inhibitory effect on EYA1 or PAX2 translation or activity. These findings raise the possibility that SLIT2/ROBO2 signaling controls such posttranscriptional effects on EYA1 and/or PAX2. Consistent with this possibility, it has been suggested that SLIT and ROBO are required to promote an asymmetric cell division during *Drosophila* neural development via a negative effect on Nubbin and Mitimere protein levels (Mehta and Bhat, 2001). Additional studies will be needed to define the mechanism by which SLIT2/ROBO2 signaling interacts

with the GDNF and perhaps other signaling systems to produce the single UB that invariantly forms at a specific time and place in the normal embryo.

#### Experimental Procedures

##### Mouse Mutant Analysis

The *Slit2* (Plump et al., 2002) and *Gdnf* (Moore et al., 1996) null alleles and the *Hoxb7*-GFP transgene (Srinivas et al., 1999) were maintained on mixed genetic backgrounds. Normal animals were either wild-type or heterozygous littermates of the *Slit2* or *Robo2* mutants or were offspring of outbred CD1 mice (Charles River Laboratories). Embryos were staged by counting somites as previously described (Sun et al., 2000).

##### Gene Expression Analysis and Detection of Cytokeratin

RNA in situ hybridization analysis on cryosections and in whole mount were performed according to standard protocols, using probes for mouse *Slit2*, *Gdnf*, *Pax2*, *Eya1*, and rat *Robo2*. For whole-mount immunofluorescence with mouse anti-pan cytokeratin antibody (Sigma C9687), the urogenital system was isolated at E11.5 and E14.5, fixed in cold methanol, and stored at  $-20^{\circ}\text{C}$  before processing according to standard protocols.

##### Production and Analysis of *Robo2* Mutant Mice

The targeting vector used to produce the *Robo2* null allele in ES cells was constructed using  $\sim 6.1$  kb of *Robo2* genomic DNA isolated from a BAC mouse ES cell library (Release II; Incyte Genomics) and electroporated into E14Tg2a.4 ES cells, derived from the 129/Ola strain (Mitchell et al., 2001). Three homologous recombinants (out of 74 clones assayed) were identified by Southern blot analysis. Germline transmission of the *Robo2* mutant allele was obtained with one of these clones. The mutant allele was maintained on a mixed genetic background (CD1; C57/BL6; 129Sv). Animals were genotyped using a PCR assay: for the wild-type allele, we used an antisense strand primer (5'-CATTCGGGGTCTCTCTTAGCATTGTT-3') and a sense strand primer (5'-TCATATGTCAAATGAATCCTCTGATGTTTTAC-3') to amplify a 290 bp fragment; for the mutant allele, we used the same antisense strand primer and a sense strand primer (5'-GAAGAAGGCACATGGCTGAATATCG-3') to amplify a 1.4 kb fragment.

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