

# To bud or not to bud: the RET perspective in CAKUT

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**Abstract** Congenital anomalies of the kidneys or lower urinary tract (CAKUT) encompass a spectrum of anomalies that result from aberrations in spatio-temporal regulation of genetic, epigenetic, environmental, and molecular signals at key stages of urinary tract development. The Rearranged in Transfection (RET) tyrosine kinase signaling system is a major pathway required for normal development of the kidneys, ureters, peripheral and enteric nervous systems. In the kidneys, RET is activated by interaction with the ligand glial cell line-derived neurotrophic factor (GDNF) and coreceptor GFR $\alpha$ 1. This activated complex regulates a number of downstream signaling cascades (PLC $\gamma$ , MAPK, and PI3K) that control proliferation, migration, renewal, and apoptosis. Disruption of these events is thought to underlie diseases arising from aberrant RET signaling. *RET* mutations are found in 5–30 % of CAKUT patients and a number of *Ret* mouse mutants show a spectrum of kidney and lower urinary tract defects reminiscent of CAKUT in humans. The remarkable similarities between mouse and human kidney development and in defects due to *RET* mutations has led to using RET signaling as a paradigm for determining the fundamental principles in patterning of the upper and lower urinary tract and for understanding CAKUT pathogenesis. In this review, we provide an overview of studies in vivo that delineate expression and the functional importance of RET

signaling complex during different stages of development of the upper and lower urinary tracts. We discuss how RET signaling balances activating and inhibitory signals emanating from its docking tyrosines and its interaction with upstream and downstream regulators to precisely modulate different aspects of Wolffian duct patterning and branching morphogenesis. We outline the diversity of cellular mechanisms regulated by RET, disruption of which causes malformations ranging from renal agenesis to multicystic dysplastic kidneys in the upper tract and vesicoureteral reflux or ureteropelvic junction obstruction in the lower tract.

**Keywords** RET · GDNF · CAKUT · Wolffian duct · Kidney development · Kidney anomalies · Ureter anomalies

## Introduction

Mutations in Rearranged in Transfection (*RET*) are associated with several diseases including congenital anomalies of the kidneys or urinary tract (CAKUT), Hirschsprung's disease, and multiple endocrine neoplasia type 2 (MEN2) syndromes. Loss-of-function germline mutations in *RET* are associated with Hirschsprung's disease in 30–70 % of patients [1]. Germline gain-of-function *RET* mutations are associated with cancer syndromes: MEN2A, MEN2B, and familial medullary thyroid cancer [2]. Somatic *RET* mutations have been linked to pheochromocytoma and sporadic medullary thyroid cancer. *RET* gene rearrangements are a cause in a subset of papillary thyroid carcinomas and lung cancers [3]. It is reported that approximately 5 % of living patients with CAKUT harbor mutations in the RET pathway [4], and *RET* mutations are present in 7 % [5] of fetuses with CAKUT, and in 30 % of fetuses with unilateral or bilateral renal agenesis [6]. The pleiotropic roles of RET in several organ systems in humans has generated considerable interest in understanding the

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molecular mechanisms of RET function within and across different systems.

Almost two decades ago, RET was found to be required for kidney development. Mice with absent Ret signaling did not develop kidneys and therefore died at birth [7]. Since then the urinary tract of the mouse has been a model system to study RET's physiological roles during development and disease. New genetic tools are providing key insights into (1) the basis for redundancy and specificity during urinary tract development, (2) determining interactions between *RET* and other genes important in kidney development, and (3) determining the roles of RET in renal homeostasis throughout embryonic development as well as postnatally. The insights from recent and ongoing studies will potentially lead to the discovery of biomarkers and therapeutic targets for congenital renal disease, acute kidney injury, and cancer treatment. Here, we will discuss the current knowledge of RET signaling in kidney development and CAKUT with a focus on insights derived from studies in vivo using mice with aberrant Ret signaling.

### RET structure

*RET* was first discovered as a gene rearrangement in fibroblast cell culture [8]. The *RET* gene is located on chromosome 10q11.2 in humans. It consists of 20 exons and encodes a single-pass transmembrane protein that exists as two main isoforms with a molecular mass of 150 and 170 kDa, due to alternative splicing in the carboxy-terminus (Fig. 1a). The long isoform (RET51) harbors 1115 aa and the short isoform (RET9) harbors 1072aa [9]. RET is a member of the receptor tyrosine kinase (RTK) family [10]. The extracellular region consists of four cadherin-like domains and a cysteine-rich domain that mediates co-receptor and ligand binding. The cytoplasmic portion has two kinase domains with specific tyrosine residue phosphorylation sites which upon activation trigger downstream signaling [11].

### RET signaling components

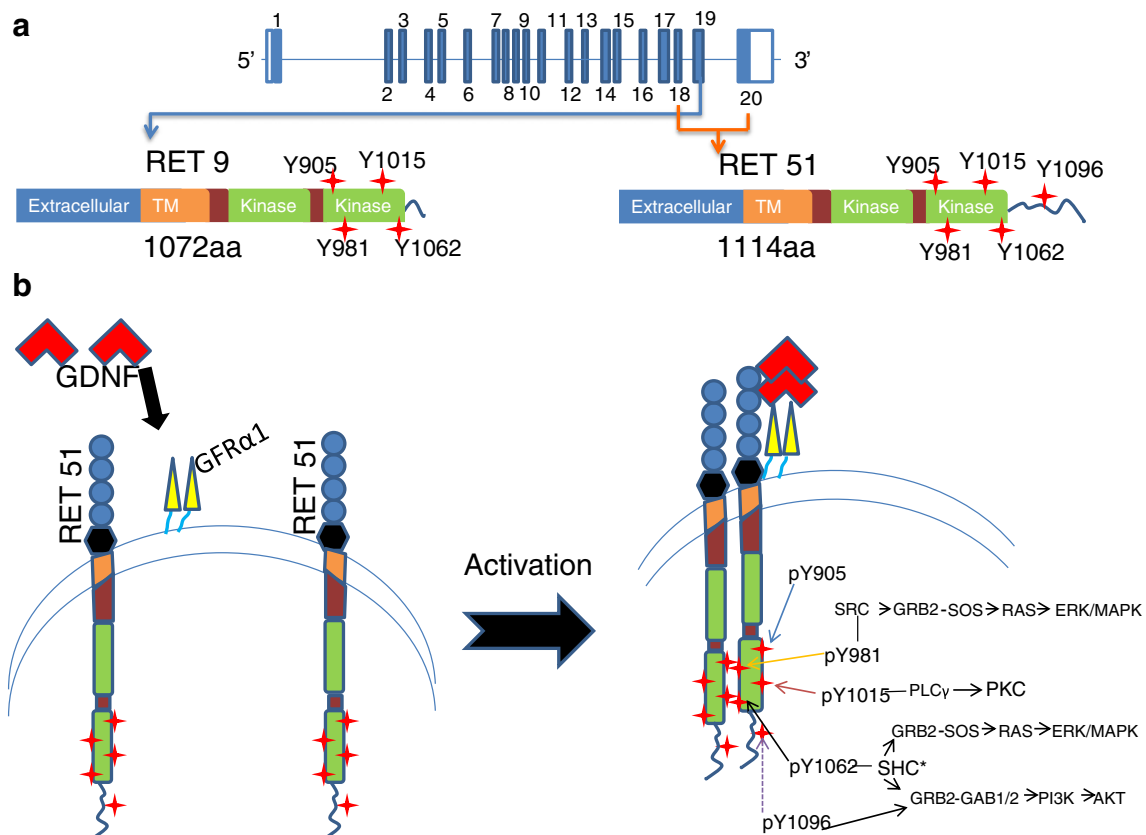
RET is activated by its interaction with one of the four members of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) and one of the four GFL coreceptors (GFR $\alpha$  1–4). The four GFLs are GDNF, Neurturin, Artemin, and Persephin. Each GFL preferentially binds one of the GFR $\alpha$  coreceptors to activate RET, although some cross-reactivity exists. Since GDNF and GFR $\alpha$ 1 are the physiologically relevant partners of RET in the urinary tract we will focus on GDNF-GFR $\alpha$ 1-RET signaling. GDNF is a secreted glycoprotein related to the transforming growth factor  $\beta$  superfamily and is primarily expressed in the mesenchymal compartment of the urinary system. In contrast, RET is

expressed only in the urinary tract epithelium [12]. RET is the only known RTK that does not bind directly to its ligand. It requires a co-receptor for activation. Interestingly, the co-receptor, GFR $\alpha$ 1, is expressed in both the mesenchyme and the urinary tract epithelium [13–16].

The current view is that the activated GDNF-GFR $\alpha$ 1-RET complex is a hexameric structure consisting of homodimers of GDNF, GFR $\alpha$ 1 and RET [17]. Formation of this multimeric complex results in phosphorylation of specific tyrosine residues in the cytoplasmic domain (Fig. 1b). The major tyrosine (Y) residues are Y981, Y1015 and Y1062 and they serve as interacting sites for intracellular adaptor proteins that activate downstream signaling cascades [9, 17]. The residue Y1015 is a docking site for phospholipase C- $\gamma$  (PLC $\gamma$ ) and activates the protein kinase C (PKC) pathway; Y981 binds the adaptor SRC and activates MAPK cascade, and Y1062 can bind multiple docking proteins including SHC, IGF, FRS1 and FRS2 (please note in Fig. 1b only SHC is illustrated for simplicity). Activated SHC recruits either the adaptor complex GRB2-GAB to activate the PI3K-AKT pathway or the GRB2-SOS complex to activate the ERK-MAPK pathway. The RET51 isoform harbors an additional docking site, Y1096, for binding GRB2, which can also activate the PI3K cascade. Depending on tissue and cell type, these docking sites have redundant or unique biological roles (see later).

### Overview of kidney development

In mammals, early kidney development occurs in an anterior-posterior (cephalad-caudal) direction. Three pairs of kidneys are initiated from the intermediate mesoderm (IM) in the following order: pronephros, mesonephros, and metanephros [18, 19]. The metanephros ultimately becomes the permanent kidney. A critical event in urinary tract development is the formation of the Wolffian duct (WD), a simple epithelial tube, from a collection of cells that undergo mesenchyme-to-epithelial transition in the IM. A subset of cells in the IM also gives rise to the mesenchymal compartment adjacent to the developing WD. These structures initially constitute the first embryonic kidney, the pronephros. The pronephros is a nonfunctional lateral extension from the WD that develops in humans at about 3 weeks of gestation (embryonic day E8.5 in mice). The pronephros degenerates by 4 weeks of gestation (E9 in mice) and the next kidney, the mesonephros begins to form. The mesonephros consists of linearly arranged mesonephric tubules connected to the WD. Although functional, the mesonephros is also a transient structure and regresses in a caudal to rostral direction except the most rostral mesonephric tubules will become part of the epididymis in males. The permanent kidney, the metanephros, appears at about 4.5 weeks of gestation in humans (E10.5 in mice) as an outgrowth, called the ureteric bud (UB), from the caudal

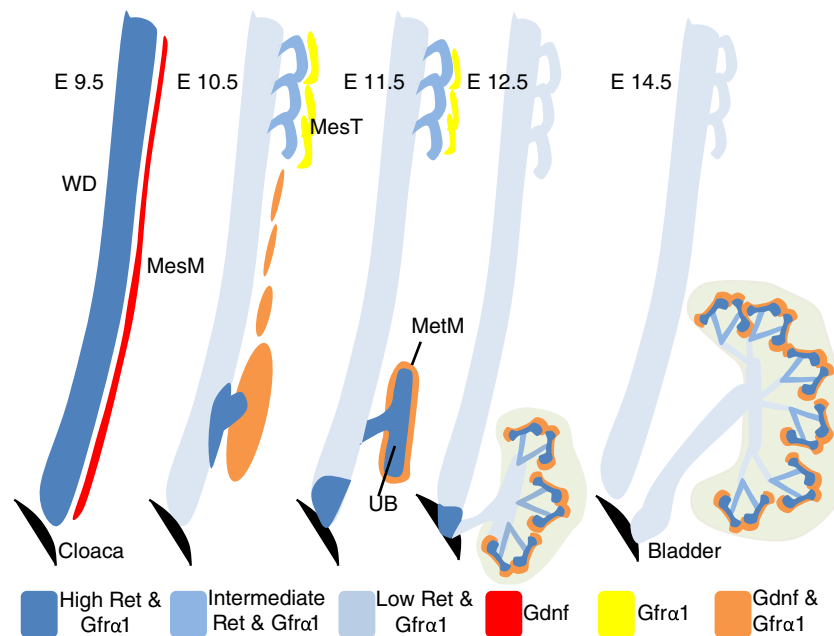


**Fig. 1** RET structure and signaling system. **a** Illustration shows *RET* gene structure with 20 exons (solid boxes) and the putative two major *RET* isoforms: short *RET* 9 (1072 aa) or long *RET* 51 (1114 aa). The different isoforms are generated by alternative splicing and differ only in the number of aa residues in the cytosolic tail. *RET* 9 has nine amino acids at the carboxyl terminus that are different than the 51 terminal residues in *RET* 51. The extracellular domain consists of four cadherin motifs. The key tyrosine (Y) residues in the cytoplasmic domain are labeled. Note that the *RET* 51 isoform has an additional tyrosine residue Y1096. **b** Schematic shows *RET* signaling components using *RET* 51 as an example. *GFRα1*, the essential co-receptor of *RET*, is a glycosphosphatidylinositol linked protein present on cell surface in the epithelia or in the

mesenchyme. Two molecules of GDNF, ligand for *RET* signaling, bind to homodimers of *GFRα1* which then recruit two *RET* molecules in the cell membrane. Formation of this hexameric complex induces autophosphorylation of specific tyrosine residues in the intracellular kinase domain that serve as docking sites for the intracellular adaptors and activates the indicated downstream signaling cascades. Asterisks on *SHC* denote that other adaptors including *SHC*, *IRS1-2*, *FRS2*, *DOK 1–6*, and *ENIGMA* can also bind to the multi-docking Y1062 site. Activated *SHC* can either trigger the *PI3K-AKT* pathway or the *ERK-MAPK* pathway depending on the adaptor complex recruited. *RET* 51 has the additional residue Y1096 which can also activate the *PI3K-AKT* pathway. (aa amino acids; *UB* ureteric bud; *PLCγ* phospholipase Cγ; *PKC* protein kinase C)

aspect of the WD that by now has reached the cloaca (future bladder). The formation of this out-pouching from the WD is believed to occur because of inductive signals from the surrounding metanephric mesenchyme (MetM) and is termed induction of the UB. The UB invades the MetM, forms an initial branch to create a T-shaped structure with two tips and a stalk. Each tip subsequently undergoes branching morphogenesis. This is a reiterative process that is dependent on reciprocal interactions between the mesenchyme and the UB. The kidney grows in a centripetal pattern with the greatest cell proliferation at the outer most edge called the nephrogenic zone (NZ). There is a high concentration of *RET*-expressing cells within UB tips. Nephrogenesis and branching morphogenesis occur concurrently and influences each other's development through a process called reciprocal interactions. Nephrogenesis is completed before birth in humans but it continues for about a week after birth in mice.

To glean insights into the function of *RET* signaling components, we and others have reported expression of *Gdnf*, *GFRα1*, and *Ret* during different stages of kidney development in mice (Fig. 2). *Ret* expression is detected as early as E8.5d in a subset of cells in the anterior WD during the pronephros and mesonephros stages. Cells that express both *Ret* and *GFRα1* are the major cell types that populate the growing WD. *GFRα1* and *Gdnf* are expressed in the adjacent mesonephric mesenchyme (MesM) at E10–10.5 and in the MetM at E11.5 [13, 20, 21]. *Ret-GFRα1*-expressing cells undergo cellular rearrangement in the WD and migrate towards the putative UB budding site at E10.5 in the mouse [22]. During UB induction, the greatest *Ret-GFRα1* expression is in the growing UB with weaker expression in the UB stalk. During branching morphogenesis *Ret-GFRα1* expression continues to be highest in the distal UB tips. The condensing mesenchyme harbors *Gdnf* and *GFRα1*-expressing cells (albeit *GFRα1* expression is much weaker than



**Fig. 2** RET, GDNF, and GFR $\alpha$ 1 expression during murine kidney development. Schematic illustrates Ret, Gdnf, and Gfr $\alpha$ 1 expression at the indicated time points during kidney development in the mouse. Ret and Gfr $\alpha$ 1 proteins are expressed along the entire WD whereas Gdnf is expressed in the adjacent MesM at E9.5 (corresponds to approximately 3 weeks in humans). At E10.5, the site of UB induction shows high Gfr $\alpha$ 1 and Ret expression compared to that in the anterior WD. Note that Gfr $\alpha$ 1 is also expressed in the MesM and MesT. The Wolffian duct

attaches to the cloaca by E10.5 and the MesM begins to degenerate. At E11.5, and during subsequent branching high expression of Ret and Gfr $\alpha$ 1 is present in the UB tips. Gfr $\alpha$ 1 and Gdnf are also expressed in the MetM and activate Ret signaling in the UB. The common nephric duct (CND) has high Ret and Gfr $\alpha$ 1 expression and undergoes apoptosis to ensure proper ureter migration and insertion into the bladder. *WD* Wolffian duct; *MesM* mesonephric mesenchyme; *UB* ureteric bud; *MesT* mesonephric tubules; *MetM* metanephric mesenchyme

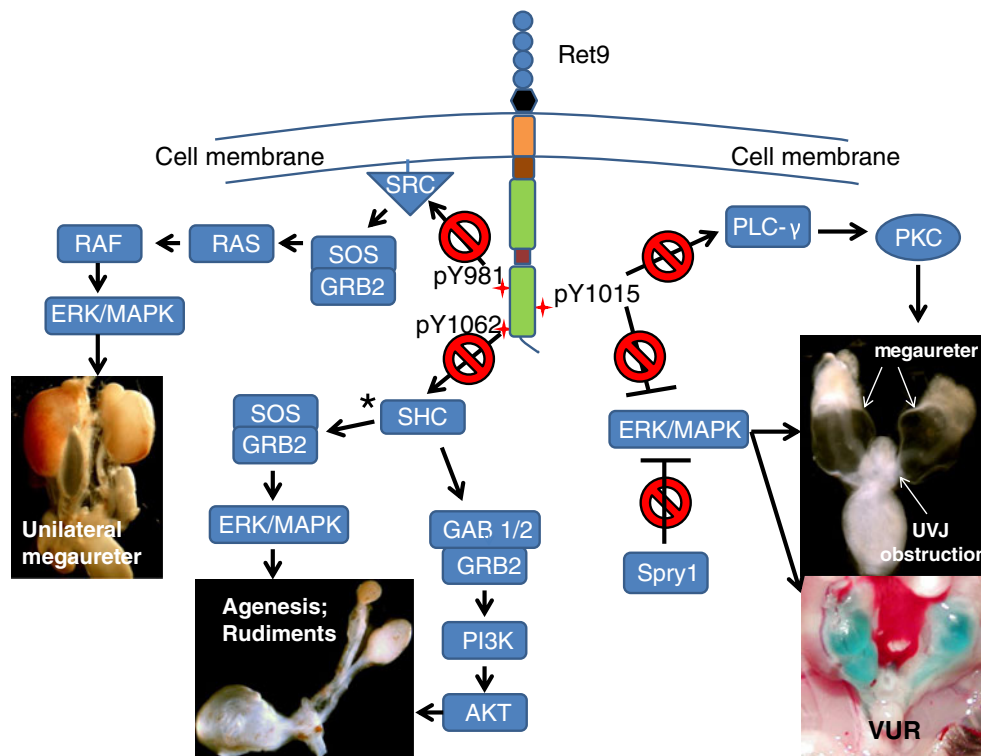
that in the UB) [13]. Ret-Gfr $\alpha$ 1 expression in the UB stalks, ureter, and the WD becomes weaker to undetectable except in the common nephric duct (CND, the distal most portion of the WD after UB induction) [20, 23]. Using Ret and Gfr $\alpha$ 1-reporter mice, we have observed Ret in a subset of cortical and medullary collecting ducts and Gfr $\alpha$ 1 in a subset of collecting ducts and proximal tubules postnatally (unpublished data).

### Physiological roles of Gdnf, Gfr $\alpha$ 1 and Ret in the kidney

Biological roles of RET signaling components in the kidney and urinary tract have been revealed by three types of experimental model systems in vivo: (1) knockout mice with complete loss of protein from beginning of gestation, (2) tissue-specific transgenic mice or mice that express mutant forms of Ret knocked-in the *Ret* locus, and (3) mice that enable conditional or tissue-specific deletion of Ret. Work of several laboratories has shown that *Ret*, *Gdnf*, and *Gfr $\alpha$ 1*-null mice die at birth due to bilateral renal aplasia or agenesis [7, 24–28]. Mice lacking GFLs other than Gdnf or lacking Gfr $\alpha$ 2–4 have normal kidneys, indicating that Gdnf-Gfr $\alpha$ 1-Ret is the relevant Ret signaling complex in the developing kidney [29–32]. Several studies show that mice with aberrant Ret expression cause a spectrum of renal anomalies, suggesting the importance of

precise regulation of Ret during development. For example, when Ret was expressed throughout the collecting system (normally localized only to the UB tips) using the *HoxB7* promoter, it resulted in multicystic dysplastic kidneys [33]. Mice expressing a dominant negative *Ret* mutation that severely diminishes ERK activity exhibited a variety of anomalies, including unilateral agenesis, bilateral hypoplasia, cysts, and bilateral agenesis [34]. More recently, knock-in mice expressing Ret isoforms or specific mutations in key docking tyrosines have provided evidence for much broader roles of Ret pathways in renal development and CAKUT [35–37]. Studies of mice expressing either wild-type Ret9 or Ret51 or Ret mutations with abrogation of adaptor sites for PLC $\gamma$  or Src or Shc reveal novel roles of Ret signaling and identify mechanisms of specificity and redundancy in kidney development (Fig. 3) [35, 37, 38]. Here, we will summarize the spectrum of renal anomalies in Ret mutant mice and then discuss the pathogenetic mechanisms that lead to these defects.

In order to study the difference between signaling by the two Ret isoforms, human cDNAs harboring individual docking tyrosine mutations of each isoform were introduced in the *Ret* locus using knockout-knock-in approach [35]. Ret docking tyrosine mutations (Y981F, Y1015F, or Y1062F) in the context of the Ret9 isoform (also lacks the Grb2 Y1096 adaptor site) in general had more severe defects than docking tyrosine mutations in the Ret51 isoform, suggesting that the



**Fig. 3** Role of specific Ret tyrosine residues and downstream signaling cascades in kidney development. Schematic showing Ret9 intracellular structure with three major tyrosine phosphorylation docking sites and their downstream targets. Loss of Y1015 signaling in either isoform of Ret prevents recruitment of PLC $\gamma$  causing severe renal dysplasia with multiplexed kidneys, UVJ obstruction, megaureter, VUR, and gonadal dysgenesis. Y1062 is a multidocking adaptor site (*asterisk*, only SHC shown, see text for other binding proteins). Loss of Y1062 in Ret9

abrogates MAPK and PI3K signaling and causes bilateral renal agenesis. The loss of signaling through the Y981 docking site affects recruitment of SRC resulting in reduced MAPK activity and causes an incompletely penetrant megaureter. Note that in the Ret51 isoform (not shown) the additional Y1096 site provides redundancy and normal kidney formation occurs when only Y981 or Y1062 are mutated. *UVJ* ureterovesical junction; *VUR* vesicoureteral reflux

C-terminus Grb2-PI3K signaling provides redundancy in kidney development [35, 38]. Mice lacking only the Src adaptor site (Ret51Y981F) had normal kidneys but a subset of those lacking the Src and Grb2 site (Ret9Y981F) displayed unilateral or bilateral kidney and ureter defects (Fig. 3). Abrogating RetY1015 activity (Y1015) results in loss of PLC $\gamma$  recruitment and bilateral megaureters, dysplastic multiplexed kidneys, and failure of the gonads to descend in both males and females revealing a novel role of Ret in genitourinary development and CAKUT (Fig. 3) [35, 39]. A similar phenotype has been reported in chimeric mice deficient for PLC- $\gamma$ 1 [40]. Abrogation of the multidocking adaptor site Y1062 (Ret51Y1062F) alone did not significantly affect kidney development. However, abrogating Y1062 and Grb2 site (Ret9Y1062F) results in bilateral agenesis or severe aplasia, a phenotype similar to that seen in Ret knockout mice (Fig. 3). Mice that express both mutant Ret9Y1062F and Ret51Y1062F have a milder phenotype: hypoplastic kidneys with reduced branching. This finding suggests that the extra Y1096 in Ret51 mice provides redundancy [35, 37, 38]. Mice with deletion of Frs2 $\alpha$  from the ureteric epithelium show mild renal hypoplasia with decreased Ret expression, suggesting that the Frs2 $\alpha$  adaptor may be one of the physiologically relevant

adaptor proteins docking at RetY1062 [41]. Finally, a recent study also showed that Ret activity can be negatively regulated by protein kinase (PKA) via a mutation of the serine phosphorylation site Ser697. Abrogation of this PKA site results in renal hypoplasia [42]. These analyses provide evidence that some of the phenotypic diversity seen in CAKUT can occur due to differential regulation of signaling cascades regulated by the same gene *RET*. In contrast to above, relative to the different isoforms of RET, it should be mentioned that a separate study using a chimeric protein with human RET in the intracellular domain and mouse Ret in the extracellular domain reported that Ret51 did not support normal kidney development [36]. The reason for the different results, one study finding that either isoform alone, RET9 or RET51, is sufficient for normal kidney development [35] versus a second study that showed mutations in RET51 resulted in loss of normal kidney development [36] remains unknown, but likely due to the different properties of human RET51 and chimeric murine Ret51.

One of the challenges in the study of Ret has been to determine the role of Ret signaling after UB budding in vivo. Based on continued Gfr $\alpha$ 1 and Ret expression during branching morphogenesis, RET signaling has been hypothesized to play a critical role during branching morphogenesis [43]. While most

of the above models investigate the role of Gdnf-Gfr $\alpha$ 1-Ret loss from the onset of emergence of renal primordia, little is known if these proteins have a role after UB induction in vivo. Experiments in vitro using kidney explant cultures exposed to Gdnf show a 1.5–2-fold increase in branching [44, 45]. Meanwhile, branching is inhibited when renal primordia are grown in the presence of Gdnf antibodies [46, 47]. To directly examine the role of the Ret signaling pathway after UB induction, we created *Gfra1* and *Ret* conditional alleles that allow Cre-mediated cell-type-specific or tamoxifen-inducible deletion of these genes after UB induction in mice [35, 48]. Interestingly, we found that the requirement of *Gfra1* depends on the stage of kidney formation. In the preUB induction stage, deletion of *Gfra1* from the WD directed by the HoxB7-Cre transgene resulted in renal agenesis or aplasia. This finding demonstrates that Gfr $\alpha$ 1 in trans from the MetM is not sufficient to compensate for its loss in the WD and that Gfr $\alpha$ 1 in the WD is required for kidney formation [13]. Surprisingly, Gfr $\alpha$ 1 deletion in the UB after induction with a tamoxifen-inducible Cre-lox system in vivo and in explant cultures had only a modest effect on overall kidney size (a 20–25 % reduction) and branching morphogenesis. This suggests that post-UB induction branching may also depend on signals other than *Gfra1* in the UB epithelium. Since *Gfra1* was not completely deleted from the MetM using the tamoxifen-inducible model, it is also possible that Gfr $\alpha$ 1 from the MetM partially compensates by activating Ret in *trans* during branching. While Gfr $\alpha$ 1 expression in the MetM is dispensable for normal kidney development [14], it remains to be seen if conditional deletion of Gfr $\alpha$ 1 from both the UB and MetM has a more severe effect on branching. Nevertheless, two important conclusions are reached from this study: (1) *Gfra1* is more critical in preUB urinary epithelium and less so post-UB induction, and (2) collecting duct progenitors do not require *Gfra1* to colonize the UB tips after UB induction. The studies with Ret and Gdnf conditional mice are in progress and will be highly informative in definitively establishing the physiological roles of the Ret signaling components at different stages of kidney development.

### The role of Ret in the lower urinary tract

The lower urinary tract consists of the ureter, bladder, and urethra. The ureter arises from the WD while the bladder and urethra arise from the cloaca and urogenital sinus. Specific cellular events are required for proper connection of the ureter to the bladder. These events have been described in several reviews [49–52]. Briefly, these include growth of the WD toward the cloaca, proper location of the UB induction site on the distal WD and degeneration of the CND. This is accompanied by a series of ureter maturation events including apoptosis of the CND, migration and tunneling into the bladder trigone forming the ureterovesical junction, and separation

of the WD from the ureter. In males, the WD becomes the vas deferens and connects to the seminal vesicle and prostate whereas in females, it becomes a rudiment. Disruption of these processes can lead to a spectrum of urinary tract anomalies such as hydronephrosis, ureterovesical junction (UVJ) obstruction, megaureter, ureterocele, and vesicoureteral reflux (VUR) [53].

Ret is critical for ureter formation and maturation because Ret-null mice harbor a number of ureter defects including no ureters, small ureters, and abnormally connected ureters to the gonadal ducts [23, 53]. Analysis of Ret-null mice show that Ret is required for proper insertion of the WD into the primitive bladder prior to UB formation [53]. The WDs of Ret-null mice either do not reach the cloaca or show a delayed fusion with the cloaca causing abnormal insertion into the bladder [53]. Mice that overexpress Ret also develop VUR [54], indicating that both loss and overexpression of Ret can cause lower tract anomalies. We recently analyzed mice with a mutation in the Ret-PLC $\gamma$  docking site (Y1015) and found that the CNDs do not undergo timely degeneration and fail to separate from the WD, resulting in obstructive nephropathy [20]. The ureter defect in RetY1015F mice is different than that in Ret-null mice as it occurs during the ureter maturation stage after the WD has reached cloaca. It remains to be determined if the Ret-null phenotype is due to collective abrogation of all Ret-activated pathways or if there is a specific signal that ensures that WD reaches the cloaca.

### Ret and other genes in the urinary system

Several genes known to be important during kidney development are upstream regulators of Gdnf-Ret signaling or are regulated by this pathway downstream (Fig. 4). Here we report a few examples of genes that affect, or are affected by, the Ret signaling complex.

#### Upstream regulators of Gdnf-Ret signaling in vivo

Characterization of a number of mice with mutations of key kidney development genes show similar phenotypes to those seen in mice with *Gdnf-Gfra1-Ret* mutations. Functional analysis of these genetic mutations often reveals perturbations in cellular pathways that are upstream or downstream of the Ret signaling pathway. For instance, conditional deletion of *Rar* results in hypoplastic kidneys or agenesis due to transcriptional downregulation of Ret [55]. *Rar* and *Gata3* signaling modulate Ret expression and thereby regulate the joining of the WD to cloaca [53]. *Bmp4*-null mice show increased Gdnf expression and extra UBs indicating that *Bmp4* is a negative regulator of Gdnf expression and Ret signaling [56]. *Pax2*-null mice show severe reduction in mesonephros development and reduction in Gdnf expression [57]. Slit2 and

Robo2-null mice show expansion of the Gdnf-expression domain, suggesting that these proteins negatively regulate Gdnf expression and inhibit Ret [58].

Downstream proteins interacting with Gdnf-Ret signaling in vivo

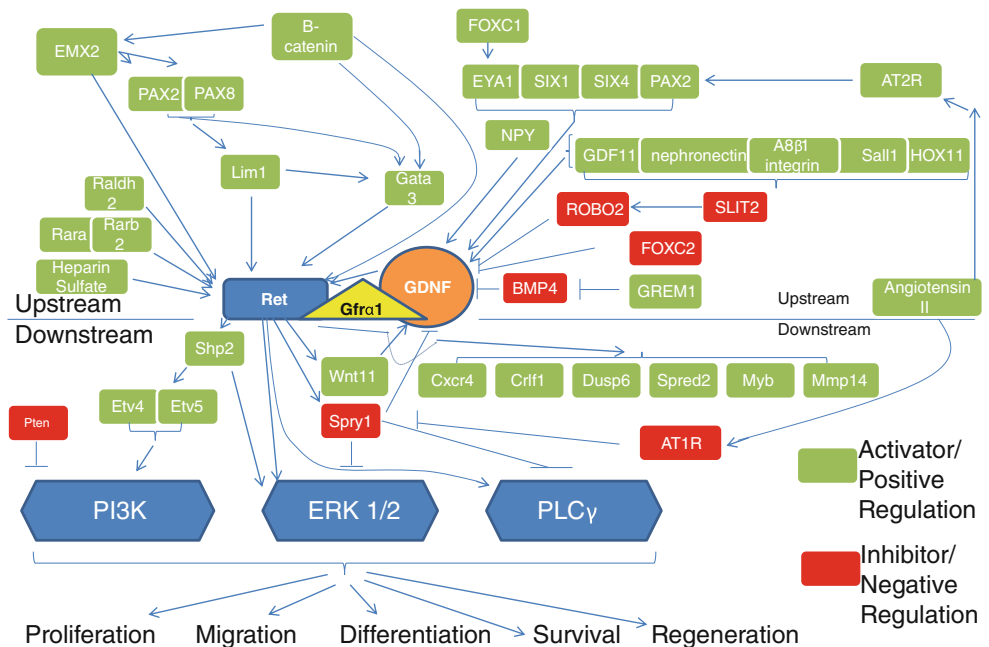
Activity of many genes is modulated by Ret signaling. Ret regulates the expression of Wnt11, Etv4, and Etv5 [44, 59]. Further, *Wnt11*-null and *Etv4*-*Etv5* double-null mice show renal hypoplasia or agenesis, phenotypes similar to those seen in a subset of *Ret*-mutant mice. Mice that are null for Shp2, a tyrosine phosphatase, also have hypoplastic kidneys [45]. These mice exhibit down-regulation of Wnt11, Etv4, and Etv5, indicating that Shp2 is a downstream modulator of RET signaling [45]. A phenotype similar to Y1015F ureter defects was described in mice deficient for leukocyte antigen-related (Lar) family protein tyrosine phosphatase, receptor type S and F (Ptpns and Ptpfr). Increased activation of Ret-docking tyrosines in these mice suggests an interaction between Lar and Ret signaling pathways [60].

Gdnf-Ret and Spry1 interactions

Sprouty1 protein (Spry1) inhibits RTK signaling by suppressing RAS-MAPK activity (Figs. 3 and 4) [61]. Therefore, a *Spry1*-null mutation results in hyperactivation of the RAS-MAPK cascade [62]. *Spry1*-null mice show multiplexed dysplastic kidneys with megaureters and supernumerary UBs [62]. This is in contrast to the renal agenesis phenotype in the *Ret*-null or *Ret*-Y1062F mutant mice where UB induction is inhibited due to severe reduction in RAS-MAPK signaling.

These observations suggest *Spry1* is a negative regulator of UB development. *Spry1*-nulls and *Ret*-nulls represent extreme forms of CAKUT that emerge from dysregulation of MAPK signaling. Several laboratories performed experiments to test genetic interactions between these proteins in vivo. Double homozygous mutant mice that lack Ret-PI3K-MAPK signaling and *Spry1* (*Spry1*<sup>-/-</sup> : *Ret*<sup>Y1062F/Y1062F</sup>) surprisingly showed morphological correction of the disparate renal anomalies seen in these individual null mice [63]. This remarkable correction of two different phenotypes has potential implications in modulating these pathways in CAKUT therapy. The mechanism for normalization of the renal defects was postulated to be through an increase in RAS-MAPK signaling via a pathway other than Ret. A likely candidate for this was FGFR signaling as in the absence of Gdnf/Ret signaling FGF7 can induce UBs [64]. Further, support for alternative tyrosine kinase activity comes from compound homozygous mice lacking Gdnf or Ret, and *Spry1*. Mice lacking Gdnf and *Spry1* (*Spry1*<sup>-/-</sup> : *Gdnf*<sup>-/-</sup>) or Ret and *Spry1* (*Spry1*<sup>-/-</sup> : *Ret*<sup>-/-</sup>) surprisingly did not show the renal agenesis or complex CAKUT seen in *Ret*-null or *Spry1*-null mice, respectively. These double mutant mice develop mildly hypoplastic kidneys (~70 % of size of controls) almost 90 % of the time. This suggests that if basal MAPK suppression is removed (no *Spry1*), UB budding and kidneys can be induced without Ret. Removing *Fgf10* from *Ret*-*Spry1* double nulls results in no UBs, suggesting that *Fgf10* presumably through activation of the receptor *Fgfr2* was the alternative RTK signal for UB induction [65]. The ampullae of the *Ret*-*Spry1* or *Gdnf*-*Spry1* double mutant mice are dilated, suggesting that Ret may be still required to regulate some aspects of branching. Because the kidneys are essentially of normal size

**Fig. 4** Gene and pathways associated with the RET signaling complex. The schematic depicts the major direct and indirect regulators of RET-GFRα1-GDNF signaling (upstream) and those that are affected by RET-GFRα1-GDNF signaling (downstream). In the upstream panel, red indicates inhibitors and green indicates stimulators or activators of RET signaling. In the downstream panel, proteins in red inhibit GDNF-RET activity and those in green are activated or are positively regulated by GDNF-RET either at transcription or posttranslational level



**Table 1** Summary of RET signaling during early kidney development

Process	Ret signaling deficit	Phenotype	Mechanism
Pre UB induction	Ret null	WD unable to reach or delayed attachment to the cloaca	Arrested migration of WD
	Abrogated Ret Y1015 signaling	Delayed degeneration of MesM; Ectopic GDNF expression	Decreased apoptosis in MesM and WD; increased ERK1/2 activity
UB induction	Ret, Gdnf, Gfrα1 null	Renal agenesis or aplasia	Loss or delayed UB induction; Ret expressing cells do not localize to the site of UB induction; decreased ERK activity
	Abrogated Ret Y1062 signaling on the short isoform (no compensation/redundant signaling from Y1096 docking site)		Loss of UB induction
	Abrogated Ret Y1015 signaling	Multiple UB induction sites; Multiplexed cystic dysplastic kidneys; multiple ureters	Decreased apoptosis of the MesM; Broadened area of MetM expression; loss of proper demarcation of the WD and MetM; increased ERK activity
Branching morphogenesis	Loss of Gfrα1 expression only in the WD	Renal agenesis	No trans-signaling by Gfrα1 in mesenchyme
	Loss of Gfrα1 only in the mesenchyme	Normal kidneys	Redundant expression in UB epithelium
	Loss of Ret or Gdnf and Spry1- (Ret null/Spry1 null) or (Gdnf null/Spry1 null)	Kidneys form at 70 % the size of control	Parallel signaling through Fgf10-Fgf2r pathway
	Loss of Ret or Gdnf and Spry1- (Ret null/Spry1 null) or (Gdnf null/Spry1 null)	30 % reduction in kidney size compared to control. Dilated ampulla tips	Ret signaling remains responsible for distal tip morphology.
	Loss of Gfrα1 from UB epithelium after UB induction	20 % reduction in kidney size compared to control. Dilated ampulla tips	Decreased proliferation and ERK 1/2 activity in cells without Gfrα1 expression.
Ureter maturation	Ret null	WD cannot reach cloaca	Arrest of migration of the WD to the cloaca
	Abrogated Ret Y1015 signaling	UVJ obstruction or VUR	Delayed degeneration of the CND due to decreased apoptosis and gain of ERK 1/2 activity

*MesM* mesonephric mesenchyme, *WD* Wolffian duct, *UB* ureteric bud, *MetM* metanephric mesenchyme



in *Spry1*<sup>-/-</sup>: *Ret*<sup>Y1062F/Y1062F</sup> mice compared to double-null *Spry1*<sup>-/-</sup>: *Gdnf*<sup>-/-</sup> or *Spry1*<sup>-/-</sup>: *Ret*<sup>-/-</sup> mice, it is possible that Ret signals other than those from Y1062 may also have a role in UB branching.

### Developmental and molecular mechanisms of kidney and urinary tract regulation by Ret

The phenotypes observed in different *Gdnf*, *Gfrα1*, and *Ret* mutants represent an allelic series that are reminiscent of CAKUT in humans. Early developmental analysis of Ret-tyrosine docking site mutants and null mice reveal that Ret signaling regulates WD morphogenesis and kidney development at four stages: (1) PreUB induction, (2) UB induction, (3) branching morphogenesis, and (4) ureter maturation (Table 1). Further, Ret has dual roles in the urinary tract. Normal Ret signaling maintains a balance between inhibitory signals mediated through Ret-Y1015 and activating signals through RetY1062. In the anterior WD, RetY1015 signaling suppresses ectopic UBs in the mesonephros by ensuring timely degradation of the surrounding mesonephric mesenchyme by apoptosis before primary UB budding and branching morphogenesis takes place [20]. In RetY1015F mutant mice, the MesM degeneration is delayed, leading to sustained exposure of the anterior WD to *Gdnf* that promotes supernumerary buds. These buds merge with the growing metanephric kidney and result in multiplexed, dysplastic kidneys. During UB induction, we also found that Ret, through Y1015, keeps a distinct boundary between the MetM and the WD. Disruption of Y1015 signals results in MetM abutting the WD in an extended UB budding domain, which provides a further stimulus for multiple UBs. In *Ret*-null mice, the UB either fails to initiate or grows into the MetM. This defect results in agenesis, hypoplasia, or aplasia. Thus, proper UB induction is under precise regulation by activating and inhibitory signals. *Ret*-null mice also show failure of the WD to make contact with the cloaca by E10.5 due to slow or arrested distal WD growth. In contrast, in RetY1015F mutant mice, the WD reaches the cloaca but does not undergo timely CND apoptosis between E12.5 and E14.5. This results in ureters that are ectopically inserted or that have a blind ending and fail to separate from the WD, leading to megaureters [20]. The mechanism of this is sustained ERK activity, which promotes proliferation and inhibits apoptosis. This suggests that Ret has a dual role in distal WD growth and maintains a balance between activating and inhibiting signals to prevent ureter abnormalities. In elegant experiments where the WD consists of chimeric *Ret*-null and wild-type cells, it was recently shown that Ret signaling is critical in controlling cell movements in the primary UB budding site [22]. *Ret*-deficient cells have delayed migration towards the primary UB budding and this finding explains the UB induction defects that result in hypoplasia or agenesis. The mechanism of hypoplasia when *Gfrα1* is conditionally deleted from the UB

epithelium after UB induction (E11.5–12.5) is decreased branching due to a reduction in UB tip cell proliferation and ERK activity [13]. These studies also show that the adjacent wild-type UB tip cells have a remarkable capability to compensate for *Gfrα1* loss.

### GDNF, GFRα1, and RET mutations in patients with CAKUT

We recently reported a study of 122 living patients, encompassing various CAKUT, and found *GDNF* or *RET* variations in about 5 % of the patients [4]. These patients are living, therefore these mutations were not deleterious enough to cause lethality, but occur in a context that affects RET signaling in combination with other interacting genes. For example, we detected rare and novel variants in *GDNF* and *RET* that coexist with a common polymorphism. Biochemical characterization showed that the presence of a common SNP *RETG691S* and a mutation *RET982* in the same allele together caused a severe reduction in MAPK signaling. Targeted capture and whole-exome sequencing identified a novel *GFRα1* mutation, in addition to the *RET-G691S* and *RET982*, in a child with complex renal anomalies including megaureters, dysplasia, and cryptorchidism. This suggests that the net burden of common and rare/novel deleterious variants in the same gene or its interacting partners determines the output from key signaling cascades and ultimately determines if anomalous renal development will occur.

### Conclusions

About 5–30 % of CAKUT patients (from analysis of about 250 patients from three studies) harbor *GDNF*, *GFRα1*, or *RET* mutations, making this pathway highly relevant to renal malformations. The analyses of *Gdnf*, *Gfrα1*, and *Ret* allelic series of mice reveal the importance of this pathway in regulating various aspects of early kidney and urinary tract development. CAKUT are genetically heterogeneous disorders—many genes interacting together are responsible for these defects. This means that co-expression of mutant forms of genes in the same pathway can exacerbate CAKUT or result in apparently normal kidneys. Several of these studies show that kidney and urinary tract development is sensitive to precise timing and net output of MAPK activity. This can be regulated by altering the dosage of the Ret signaling complex, or activity of key docking tyrosine of Ret, or by interactions with negative regulators such as *Spry1*. While significant advances have been made in RET biology, many exciting challenges and new questions have emerged.

The RET pathway is a key regulator of kidney size and nephron endowment [66], and decreased nephron number at

birth is related to hypertension and CKD [67]. An unanswered question is if variations in kidney development genes such as *RET*, that are not lethal, are risk factors or predictive of CKD progression, essential hypertension, or ESRD. It is also time to explore the contribution of structures outside the urinary tract that may be regulated by *RET* and affect the kidney. For example, *RET* shows strong expression in the autonomic and sensory nervous systems, but we do not know how *RET* activity in these systems influences kidney and urinary tract pathophysiology. Recently, GFLs were reported to be highly expressed in immunosuppressed transplant patients, suggesting a possible role in the immune system [68]. Genes important in development may also play a role in repair and regeneration after injury. Whether mutations in the *RET* pathway render one more susceptible to chemical, hypoxic, toxic, or obstructive insults in the kidney needs to be investigated. There are exciting times ahead in *RET* translational and basic research that will continue to advance our understanding of congenital, acute, and chronic kidney diseases. With interdisciplinary efforts and collaborations between basic scientists, geneticists, and clinicians and commitment of funding agencies, the field of *RET* signaling will reach new heights in the coming years.

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