Genetic mosaic analysis reveals a major role for frizzled 4 and frizzled 8 in controlling ureteric growth in the developing kidney

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SUMMARY
The developing mammalian kidney is an attractive system in which to study the control of organ growth. Targeted mutations in the Wnt receptors frizzled (Fz) 4 and Fz8 lead to reduced ureteric bud growth and a reduction in kidney size, a phenotype previously reported for loss of Wnt11. In cell culture, Fz4 and Fz8 can mediate noncanonical signaling stimulated by Wnt11, but only Fz4 mediates Wnt11-stimulated canonical signaling. In genetically mosaic mouse ureteric buds, competition between phenotypically mutant Fz4⁻/⁻ or Fz4⁺;Fz8⁻/⁻ cells and adjacent phenotypically wild-type Fz4⁺ or Fz4⁻/⁻;Fz8⁺ cells results in under-representation of the mutant cells to an extent far greater than would be predicted from the size reduction of homogeneously mutant kidneys. This discrepancy presumably reflects the compensatory action of a network of growth regulatory systems that minimize developmental perturbations. The present work represents the first description of a kidney phenotype referable to one or more Wnt receptors and demonstrates a general strategy for revealing the contribution of an individual growth regulatory pathway when it is part of a larger homeostatic network.

KEY WORDS: Fz4, Fz8, Kidney development, Wnt11, Wnt signaling, Organ size control, Mouse

INTRODUCTION
Multicellular organisms are characterized not only by the appropriate specification and spatial arrangement of diverse cell types but also by precise control of their relative abundances. At a macroscopic level, the latter feature is reflected in a match between overall body growth and the growth of individual organs and the substructures within those organs. Derangements in the control of cell proliferation that manifest as cancer generally require a large number of genetic alterations, providing evidence for a complex web of redundant and cross-regulating control systems (Weinberg, 2006). In the context of normal development, genetic perturbations that alter the rates of cell proliferation or death have been most sensitively revealed by situations in which the normal and modified cells compete for the same niche. Examples include competition between wild-type (WT) and minute cells in Drosophila imaginal discs (Martin and Morata, 2006; Neto-Silva et al., 2009), between cells with different active X-chromosomes in females heterozygous for X-linked dyskeratosis congenita (Vulliamy et al., 1997; Migeon, 1998), and between WT and severe combined immunodeficiency (SCID) cells in a bone marrow transplantation model (Otsu et al., 2000). Although the major regulators of cell proliferation, including the FGF, EGF, Hedgehog, TGFβ, Wnt and Hippo pathways, are now relatively well defined, the mechanisms by which these pathways interact to precisely regulate organ size are still largely unexplored.

The developing mammalian kidney is an attractive system in which to study epithelial-mesenchymal interactions, the local determination of cell fates and the control of cell proliferation (Dressler, 2009). The ratio of kidney size to body size critically affects renal function and homeostasis, as clinical studies indicate that even a small reduction in nephron number significantly increases the risks for renal failure and hypertension (Brenner et al., 1997; Giral et al., 2010). The earliest events in kidney development are the invasion of the metanephric mesenchyme by the ureteric bud and the subsequent growth and branching of the bud epithelium. Gdnf, a major regulator of bud growth, is produced by the mesenchyme and acts on the bud epithelium via the Ret tyrosine kinase receptor and Gfra1 co-receptor (Hellmich, 1996; Costantini and Shakya, 2006). Gdnf signaling is regulated at multiple levels. Sprouty 1 (Spry1), an intracellular kinase antagonist, inhibits Ret signaling in the bud epithelium (Chi et al., 2004; Basson et al., 2006). Wnt11, produced by the bud, increases Gdnf transcription, which, in turn, activates Wnt11 transcription (Majumdar et al., 2003). Two other Wnts, Wnt4 and Wnt9b, play major roles in the mesenchymal-to-epithelial transition that is required for the formation of renal vesicles (Stark et al., 1994; Carroll et al., 2005). Angiotensin 2, BMP, FGF, retinoic acid (RA) and TGFβ signaling pathways also regulate branching morphogenesis and growth by the ureteric bud (Godin et al., 1999; Oxburgh et al., 2004; Grieshammer et al., 2005; Bates, 2007; Dressler, 2009; Rosselot et al., 2010; Song et al., 2010).

Wnt ligands act through multiple families of receptors and co-receptors to induce changes in gene transcription (canonical Wnt signaling) or in the cytoskeleton (noncanonical Wnt signaling) (van Amerongen and Nusse, 2009). The canonical Wnt signal funnels through β-catenin, but noncanonical Wnt signaling involves pathways that are still poorly understood. In mammals there are ten seven-pass Frizzled receptors and two single-pass Lrp co-receptors. Frizzled proteins can mediate both canonical signaling in collaboration with Lrp co-receptors and noncanonical signaling,
although it appears likely that some Frizzleds mediate only one or the other type of signaling. Wnts can also signal through the Ryk and ROR transmembrane tyrosine kinase receptors. At present, the receptors that mediate Wnt signaling in kidney development are unknown, and the signaling pathways that they activate are poorly defined.

In the present work, we describe a phenotype of reduced ureteric bud growth and a reduction in kidney size in the absence of Frizzled (Fz; or Fzd) 4 and Fz8. This phenotype, together with cell culture analyses of Wnt11 signaling via Fz4 and Fz8, suggests that these two receptors might mediate some or all of the actions of Wnt11 in the mouse kidney. Using genetically mosaic buds, we further show that when phenotypically mutant Fz4+/− or Fz4−/−;Fz8−/− cells compete with adjacent phenotypically WT Fz4+/+ or Fz4+/+;Fz8+/+ cells, respectively, the mutant cells become under-represented to a far greater extent than would be predicted from the more modest growth retardation of non-mosaic kidneys. This work presents the first description of a kidney phenotype that is referable to the genetic ablation of one or more Wnt receptors, and it indicates that Frizzled signaling is a major regulator of kidney size during development. The genetic mosaic experiment also demonstrates a general strategy for revealing the contribution of individual growth regulatory pathways when they are part of a larger homeostatic network.

MATERIALS AND METHODS

Production of Fz8 knockout mice

The Fz8 knockout (KO) allele was created by standard gene-targeting methods (see Fig. S1 in the supplementary material). The targeting vector was introduced by electroporation into R1 embryonic stem (ES) cells and plated in medium containing G418 and ganciclovir. ES colonies were screened for the correct targeting event by Southern blot hybridization with flanking probes, and targeted cells were injected into C57BL/6 blastocysts. Microarray hybridization and data analysis

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complete coverage of known and predicted mouse protein-coding genes. Microarray data were analyzed with Spotfire software. Microarray data are deposited at Gene Expression Omnibus under accession numbers GSE23781 and GSE26668.

**Statistics**

P-values were calculated with Student’s t-test (Microsoft Excel) or with Spotfire software for microarray data.

**RESULTS**

**Renal hypoplasia in Fz4−/− and Fz4−/−;Fz8−/− mice**

As part of a systematic analysis of Frizzled function in vivo, we generated a targeted mutation in the mouse Fz8 gene by replacing its open reading frame with an *E. coli* β-galactosidase (lacZ) open reading frame and PGK-neo selection cassette (see Fig. S1 in the supplementary material). Since Fz8−/− mice show no defects in viability, size or fertility, we explored the possibility that Fz8 might function redundantly with one or more of the nine other Frizzled genes. In keeping with this idea, we observed that Fz4−/−;Fz8−/− mice exhibit postnatal lethality with 100% penetrance, with no Fz4−/−;Fz8+ + weanlings among 141 postnatal day (P) 14 animals, in which this genotype should have constituted 25% of the mice. By contrast, when litters from the same crosses were harvested at E18.5, Fz4−/−;Fz8−/− fetuses were found at the expected Mendelian frequency with no outward phenotypes.

Fz4 and Fz8 showed nearly identical patterns of expression within the developing kidney as determined by expression of a lacZ reporter inserted at each locus (Fig. 1A–G). Both genes were expressed in the developing ureteric buds as early as E11.5, and expression was observed not only in the collecting system, but also in the nephron epithelia until at least E15.5. After this time point, endogenous β-galactosidase enzyme activity within the kidney partially obscured the histochemical analysis of gene-targeted β-galactosidase activity. A second Fz4 allele, in which the human placental alkaline phosphatase (AP) open reading frame replaces the Fz4 open reading frame (Ye et al., 2009), revealed continuing expression of Fz4 in the collecting system and nephron epithelia as late as E18.5 (Fig. 1H,1I). These observations are consistent with whole-mount in situ hybridization analyses showing expression of Fz4 and Fz8 in the E15.5 kidney; Fz6, Fz7 and Fz10 are also expressed at this time point (McMahon et al., 2008) (www.gudmap.org). The similar patterns of Fz4 and Fz8 expression described here suggested the possibility that these genes are functionally redundant during kidney development.

At E18.5, the only gross anatomic anomaly that distinguished Fz4−/−;Fz8−/− fetuses from their control littermates was a linear reduction in kidney size of ~30% (equivalent to a ~65% reduction in proportional reduction in organ size). As part of a systematic analysis of Frizzled function in vivo, we examined the effects of kidney development in Fz4−/−;Fz8−/−;Fz6−/−;Fz7−/−;Fz10−/− fetuses with selective deletion of the Fz4 locus in the vasculature (Fz4C<sup>Cre<sup>+</sup>;Tie2Cre; Fig. 1P). Previously, we reported that Fz4 is expressed in the vasculature throughout development and that vascular defects are observed in the retina, inner ear and cerebellum in Fz4−/− mice (Xu et al., 2004; Ye et al., 2009). However, the effect of Fz4 deletion on kidney development appears to be independent of its function in the vasculature, as shown by the normal kidney size and postnatal survival of mice with selective deletion of the Fz4 locus in the vasculature (Fz4C<sup>Cre<sup>+</sup>;Tie2Cre; Fig. 1P). At present, we do not know whether renal insufficiency contributes to the postnatal lethality in Fz4−/−;Fz8−/− mice or whether there are additional defects that play a more important role.

Strikingly, the internal architecture of the smaller Fz4−/−;Fz8−/− kidneys at E18.5 appeared to be entirely normal at the light-microscope level, with morphologically normal glomeruli, proximal tubules, loops of Henle and collecting ducts, and no evidence of cysts (Fig. 1L–O). To assess nephron development in greater detail, E15.5 kidneys were analyzed by in situ hybridization for markers of nephron progenitors (Six2), pretubular aggregates (Wnt4), differentiated nephron structures including podocytes (i.e. glomeruli; Podxl), the thick ascending limb of the loop of Henle and early distal convoluted tubules (uromodulin), proximal convoluted tubules (Slc34a1) and distal convoluted tubules (Slc12a3). For each of these structures, the size, location and packing density appeared to be normal in the Fz4−/−;Fz8−/− kidney (Fig. 2). Thus, the principal defect in the mutant kidney is a proportional reduction in organ size.

**Decreased growth, branching and cell proliferation in Fz4−/−;Fz8−/− ureteric buds**

The ureteric bud serves as an important signaling center for differentiation of the nephrons, and the number of nephrons, as well as the ultimate size of the kidney, is determined by the growth of the ureteric bud (Dressler, 2009). The Fz4−/−;Fz8−/− kidney phenotype described above suggests a deficiency in the proliferation and branching of the ureteric epithelium early in kidney development. To visualize the ureteric buds at the earliest stages in their branching, E-cadherin (a marker for ureteric epithelium) and Pax2 (a marker for metanephrine mesenchyme), and Ret and Wnt11 transcripts (markers for ureteric branch tips) were localized in whole-mount kidneys at E11.5. The E11.5 Fz4−/−;Fz8−/− ureteric bud had invaded the metanephrine mesenchyme, completed the first branching event and was only subtly smaller than the control Fz4+/−;Fz8+/− bud (Fig. 3A–F). However, by E12.5, ureteric branching was clearly retarded in Fz4−/−;Fz8−/− embryos (Fig. 3G–J).

Following the first branching event at E11.5, the ureteric epithelium proliferates extensively and undergoes many rounds of branching to form a complex ureteric tree. To determine whether the Fz4−/−;Fz8−/− ureteric branching defect was accompanied by a decrease in cell proliferation and/or an increase in cell death, we examined E15.5 kidneys by BrdU pulse labeling and anti-cleaved caspase 3 immunostaining (Fig. 4A). These analyses showed a ~1.8-fold reduction in the density of BrdU-labeled cells in ureteric bud tips in Fz4−/−;Fz8−/− compared with Fz4+/−;Fz8+/− kidneys, a difference that was statistically highly significant (Fig. 4B). By contrast, there was no statistically significant difference in the number of BrdU-labeled cells in the renal pelvis, developing collecting ducts (defined as the medullary regions of the ureteric epithelium), or cortical mesenchyme at this stage (Fig. 4B), nor was there a statistically significant difference in the extremely low rate of cell death, as assessed either in ureteric bud tips or throughout the entire kidney (Fig. 4C). The overall cell density in the bud tips, as determined from the density of DAPI-stained nuclei, was similar between genotypes (Fig. 4C). A similar experiment performed at E12.5 using a 1-hour pulse of EdU showed an extremely high density of labeled nuclei in ureteric buds and mesenchyme in Fz4−/−;Fz8−/−, Fz4+/−;Fz8−/− and Fz4+/−;Fz8+/− kidneys (see Fig. S2 in the supplementary material). Although the high density of the labeled nuclei hindered precise quantification, by visual inspection the number of EdU-labeled nuclei within the ureteric buds was not dramatically different across the genotypes at this stage.

**Genetic mosaic analysis reveals that Fz4 signaling is a major regulator of kidney size**

The retarded development of Fz4−/− and Fz4−/−;Fz8−/− kidneys described above occurs in the context of a complex network of growth regulatory signals that include Wnt, Gdnf/Ret/Gfra1,
FGF, RA and BMP pathways. If negative-feedback mechanisms exist between pathways then one might expect that the phenotypic consequences of a defect in one growth-promoting pathway could be partially masked by the compensatory activation of other pathways. To reveal the intrinsic strength of the Fz4 and Fz8 signaling pathway, we designed a tissue mosaic experiment to quantify the relative efficiencies with which genetically marked $Fz4^{-/-};Fz8^{-/-}$ versus $Fz4^{+/+};Fz8^{-/-}$ or $Fz4^{-/-}$ versus $Fz4^{+/+}$ epithelial cells populate the same developing ureteric buds.

**Fig. 1. Renal growth defects in Fz4$^{-/-}$ and Fz4$^{-/-}$;Fz8$^{-/-}$ mouse embryos.**

(A-G) X-gal staining (blue) shows expression of Fz4lacZ and Fz8lacZ in the ureteric buds at E11.5 and E12.5 (A-C), and in developing nephron epithelia and the collecting system at E15.5 (D-G). A-C show intact kidneys; D-G show 100 µm vibratome sections, with F showing a section at the cortical surface. (H,I) Alkaline phosphatase (AP) histochemistry of 100 µm vibratome sections from an E18.5 Fz4AP/+ kidney shows expression throughout the nephron epithelia and collecting system. Wild-type (WT) kidney sections processed in parallel show no staining. (J,K) Dissected urogenital tracts at E18 show that loss of Fz4 leads to a selective reduction in kidney size. The size of the Fz4$^{-/-}$;Fz8$^{-/-}$ control kidney is indistinguishable from that of the WT (as quantified in P). (L-O) The internal structure and organization of E18.5 Fz4$^{-/-}$;Fz8$^{-/-}$ kidneys (M,O) closely resemble those of the WT (L) and phenotypically WT Fz4$^{+/+};Fz8^{-/-}$ (N) kidney at both a gross level (L,M; images are at the same magnification) and at the level of cellular organization (N,O). (P) Quantification of E18 kidney lengths (upper panels) and body weights (lower panels) for crosses that generated various combinations of Fz4 and/or Fz8 null alleles. See inset diagram defines kidney length. Length and weight values are normalized to those of WT and heterozygous littermates, or, for the crosses between Fz4$^{+/+};Fz8^{+/+}$ parents (third column), to a control group consisting of all Fz4$^{+/+}$ littermates regardless of Fz8 genotype as well as Fz4$^{+/+};Fz8^{+/+}$ littermates. Red horizontal bars show the mean. Scale bars: 200 µm for A,B,L,M; 100 µm for C,N,O; 500 µm for D-I.
In this experiment, Cre-mediated recombination converts a conditional Fz4 allele with a downstream but unexpressed AP reporter coding region (Fz4\(^{CKOAP}\)) into a null allele that lacks Fz4 coding and 3' UTR sequences and expresses the AP reporter under the control of the Fz4 promoter (Fz4\(^{AP}\)) (Ye et al., 2009) (see Fig. S3 in the supplementary material). When the Fz4\(^{CKOAP}\) allele is paired with a WT Fz4 allele (Fz4\(^{CKOAP}/+\)) or a conventional Fz4 null allele (Fz4\(^{AP}/–\)), Cre-mediated recombination generates AP-expressing cells that are either phenotypically WT (Fz4\(^{AP}/+\)) or mutant (Fz4\(^{AP}/–\)), respectively. To create a fine-grained mosaic of mutant and WT cells, 4-hydroxytamoxifen (4HT) was administered at E8.5 to activate Cre-mediated recombination via a ubiquitously expressed CreER at the ROSA26 locus (R26CreER). We note that the crosses used in this experiment – Fz4\(^{CKOAP}/CKOAP\) and Fz4\(^{AP}/–\);Fz8\(^{–}/–\) – generate, on average, a 1:1 ratio of experimental and control embryos within the same litter.

At E12.5, kidneys were histochemically stained for AP, the ureteric epithelium was visualized by immunostaining for calbindin, and the distal branches with and without AP+ cells were counted (Fig. 5). As seen in Fig. 5A, cells that lack Fz4 expression were dramatically under-represented in the ureteric epithelium as judged by quantifying the number of terminal branches populated by AP+ cells. This difference, which is of high statistical significance, was seen in both Fz8\(^{+/+}\) and Fz8\(^{–/–}\) backgrounds. At the dose of 4HT used here (200 \(\mu\)g), the control kidneys showed that Fz4\(^{4\text{DTP}}\) cells account for ~50% of the ureteric epithelial cells and populate over 80% of the distal branches. The failure of Fz4\(^{4\text{DTP}}\) and Fz4\(^{4\text{DTP}}\);Fz8\(^{–/–}\) cells to populate the mosaic ureteric epithelium indicates that Fz4/Fz8 signaling is a major regulator of ureteric epithelial growth. Similar experiments in which more than 10 litters were examined following 4HT delivery at E13.5 resulted in variably smaller kidneys at E18.5, indicating an ongoing requirement for Fz4 and Fz8 function during later kidney development (data not shown).

An important assumption in the genetic mosaic experiment is that experimental and control embryos are exposed to equal levels of 4HT via the maternal circulatory system would result in equal exposure among the embryos within each litter. In several experiments, we directly examined Cre-mediated recombination in extra-renal tissues by AP histochemical staining, and, as shown in Fig. 5B and Fig. S4 in the supplementary material, similar levels of Cre-mediated recombination were found in experimental
The Fz4 protein does not reduce expression at the embryos. A second assumption in these experiments is that loss of experimental kidneys implies an absence of Fz4 expression. If this assumption is valid then the absence of AP staining in the experimental (Fz4AP/–;Fz8–/–;R26CreER) control implies a lack of AP expression in the experimental kidneys. A second test of this assumption, we directly compared the level of AP expression in Fz4AP/+ and Fz4AP/– kidneys and found no differences (Fig. 5E).

It is interesting to note that there was only a small and statistically insignificant difference in the average number of terminal ureteric branches per kidney in a comparison between the two pairs of mosaic experimental and control kidneys at E12.5 (P>0.05; Student’s t-test; Fig. 5C). The ~50% mosaicism seen in the control kidneys at E12.5 suggests that ~50% of the ureteric bud cells underwent Cre-mediated recombination in both control and experimental embryos at E8.5, and therefore that the phenotypically normal Fz4KOAP/– cells in the experimental kidneys represented only ~50% of the ureteric epithelium immediately after Cre-mediated recombination. This line of reasoning implies that, in the mosaic ureteric epithelium of the experimental kidneys, phenotypically WT Fz4KOAP/– cells exhibit an increase in proliferation relative to ureteric epithelial cells in control kidneys.

**Gene expression changes in Fz4KO;Fz8KO kidneys**

To search for potential molecular mediators of Fz4 and Fz8 signaling in kidney development, we profiled the transcriptomes of Fz4KO;Fz8KO versus Fz4WT;Fz8KO and Fz4KO versus WT kidneys at E13.5 by hybridization of three independent samples to Affymetrix MOE430 2.0 gene chips (Fig. 6). As expected, Fz4 transcripts were substantially under-represented in the Fz4KO;Fz8KO and Fz4KO samples. In the Fz4KO;Fz8KO versus Fz4WT;Fz8KO comparison, but not the Fz4KO versus WT comparison, a large number of transcripts exhibited greater than 2-fold abundance changes that were statistically significant (P<0.05).

Among the transcripts that exhibited a statistically significant decline in Fz4KO;Fz8KO kidneys were those coding for Ret, Gfra1 and Gdnf (with declines estimated by chip hybridization of ~2-fold, ~2.5-fold and ~1.5-fold, respectively; Fig. 6A). As chip hybridization generally underestimates the fold reduction for rare transcripts owing to a non-zero background of nonspecific hybridization, we independently tested the abundance of Gdnf transcripts by in situ hybridization to E11.5 Fz4KO;Fz8KO versus Fz4WT;Fz8KO kidneys, a time point chosen to reveal differences in signaling components that would be predicted to determine ureteric bud growth and branching over the subsequent 1-2 days (Fig. 6A, inset). The in situ hybridization analysis revealed the previously described pattern of Gdnf expression within the mesenchyme that surrounds the ureteric bud in Fz4KO;Fz8KO kidneys (Majumdar et al., 2003), and a large decrease in transcript abundance in Fz4KO;Fz8KO kidneys relative to Fz4WT;Fz8KO kidneys (n=4 experiments). These data suggest that part of the decrease in ureteric bud growth and branching in Fz4KO;Fz8KO kidneys could be the result of a decrease in Gdnf levels and Gdnf-induced cell proliferation. We note that any decrease in Gdnf levels secondary to loss of Fz4 and Fz8 signaling is likely to be an indirect effect, as Gdnf is produced by the mesenchyme and Fz4 and Fz8 reside in the ureteric and nephron epithelia but not in the mesenchyme.

No statistically significant changes in Gdnf, Ret or Gfra1 expression were detected in Fz4KO kidneys compared with Fz4WT control kidneys (Fig. 6B). In fact, the overall transcriptome profile of Fz4KO kidneys closely resembled that of Fz4KO/– kidneys, reflecting the relatively modest defect in kidney growth in Fz4KO embryos.

Among the other transcripts with differential abundances in Fz4KO;Fz8KO versus Fz4WT;Fz8KO kidneys, transcript 5430421B17, a ~2.9 kb RNA of unknown function, was striking for its large decline in abundance in Fz4KO;Fz8KO kidneys (Fig. 6A). This

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(Fz4KO;Fz8KO;R26CreER) and control (Fz4WT;Fz8KO;R26CreER) embryos. A second assumption in these experiments is that loss of the Fz4 protein does not reduce expression at the Fz4 promoter. If this assumption is valid then the absence of AP staining in the experimental kidneys implies an absence of Fz4KO cells. Fig. 5D shows one test of this assumption: a high dose (1 mg) of 4HT was delivered to the mother at E8.5 resulting in ~90% recombination in control (Fz4KO;Fz8KO;R26CreER) kidneys at E12.5 and causing retarded ureteric bud development with retention of substantial numbers of AP-expressing Fz4KO;Fz8KO cells in all ureteric buds in the experimental (Fz4KO;Fz8KO;R26CreER) kidneys. In a
measure the Wnt11-, Fz4- and Fz8-dependence of transcriptional activity of a luciferase reporter under the control of a minimal promoter adjacent to seven LEF/TCF binding sites (Fig. 7A) (Xu et al., 2004). These experiments showed strong activation of canonical Wnt signaling that was dependent on ligand (Wnt11), receptor (Fz4) and co-receptor (Lrp5) (Fig. 7A, left). By contrast, Fz8 was unable to mediate Wnt11-dependent canonical signaling (Fig. 7A, right). As one control for the specificity of the assay, we observed a decrease in signaling when the alternate Fz4 ligand, was substituted for Wnt11 (Fig. 7A, center). This inhibition is ligand-specific, as Fz8CRD-GPI did not inhibit Norrin/Fz4/Lrp5-mediated canonical signaling, consistent with the selectivity of Norrin (the protein product of the Norrie disease gene, Ndp), an alternate Fz4 ligand, was substituted for Wnt11 (Fig. 7A, center). As a second control, we observed a decrease in signaling when the ligand-binding cysteine-rich domain (CRD) of Fz4 was co-expressed as a GPI-anchored protein (Fz4CRD-GPI); in this context, the CRD presumably acts as a competitive antagonist (Hsieh et al., 1999) (Fig. 7A, left and center). As a second control, we observed a decrease in signaling when the ligand-binding cysteine-rich domain (CRD) of Fz4 was co-expressed as a GPI-anchored protein (Fz4CRD-GPI); in this context, the CRD presumably acts as a competitive antagonist (Hsieh et al., 1999) (Fig. 7A, left and center).

### Fig. 5. Genetically marked Fz4AP/ (i.e. Fz4+/+) cells in mosaic ureteric epithelium are rapidly outgrown by their Fz4CKOAP/– (i.e. Fz4−/−) neighbors. (A) Whole-mount E12.5 mouse kidneys with AP+ cells visualized histochemically (false-colored red) and the ureteric epithelium visualized by calbindin immunostaining (green). Four representative kidneys are shown for each genotype. Embryos were exposed to 4-hydroxytamoxifen (4HT) at E8.5 via a single 200 μg subcutaneous injection into the dorsal fat pad of the mother. To the right is a quantification of the fraction of distal ureteral branches that contain any (red) or no (green) AP+ cells. Scale bar: 200 μm. (B) Histochemical stain for AP in 300 μm sections of the heads of E12.5 experimental and control littermates showing equal extents of Cre-mediated recombination at the Fz4CKOAP allele. (C) Number of distal ureteric branches per kidney at E12.5 for the experiment in A. Mean + s.d. There is a small downward trend in the experimental groups in each pairwise comparison, but the differences between the two groups are statistically insignificant (P>0.05, Student's t-test). (D) As in A, except that 1 mg 4HT was delivered to the mother by oral gavage at E8.5, leading to ~90% Fz4+/+ recombinant cells in E12.5 ureteric buds in the control kidney (left). The kidney with Fz4+/+ cells (right) shows retarded ureteric bud growth and branching with some remaining AP+ cells. (E) E18 kidney sections stained histochemically for AP show that there is no decrease in expression of AP from the Fz4 locus in the absence of Fz4 function (Fz4+/+ kidney, right panel).

**Wnt11 as a candidate ligand for Fz4 and Fz8: canonical and noncanonical signaling**

The phenotypes of reduced kidney size with preservation of renal architecture, diminished ureteric branching and reduced abundance of Gdnf transcripts that we observed in Fz4+/−;Fz8+/− embryos closely resemble those reported for Wnt11+/− embryos (Majumdar et al., 2003). These similarities suggested that Fz4 and/or Fz8 might function as receptors for Wnt11 in kidney development.

Wnt11, along with Wnt5a, has been classified as a noncanonical Wnt based on its bioactivity in Xenopus embryos and the phenotype of the silberblick (slb) wnt11 mutation in zebrafish (Heisenberg et al., 2000; Marlow et al., 2002; Veeman et al., 2003; Ulrich et al., 2005), but its roles in mammalian kidney and heart development are also compatible with it functioning as a canonical Wnt ligand (Majumdar et al., 2003; Nagy et al., 2010). To test the latter possibility, we used the Super Top Flash (STF) cell line to measure the Wnt11-, Fz4- and Fz8-dependence of transcriptional

transcript is located on mouse chromosome 7 between 85 bp and 3 kb 3’ of the Fz4 gene and is transcribed from the same strand as the Fz4 mRNA, suggesting that it derives from a longer variant of the Fz4 transcript. Other transcripts with statistically significant abundance changes encode a highly diverse set of proteins (see Table S1 in the supplementary material).
To test whether Wnt11 can activate noncanonical Wnt signaling via Fz4 and Fz8, we measured Rho activation in transiently transfected HEK 293 cells using a pulldown assay in which Rho-GTP, but not Rho-GDP, is selectively captured by the Rho-binding domain from rhotekin fused to glutathione-S-transferase (GST; Fig. 7B). This experiment showed that Wnt11 can induce noncanonical signaling via Fz4 or Fz8. The potential for Wnt11 to activate both canonical and noncanonical signaling in the same cell suggests that these pathways might interact either positively or negatively. Indeed, earlier work on inversin has suggested that in some cells there is a reciprocal relationship between canonical and noncanonical Wnt signaling (Simons et al., 2005). To explore this possibility, we asked whether pharmacological inhibition of Rho kinase (ROCK; a downstream mediator of Rho signaling) by either of two kinase inhibitors, Y-27632 and H-1152P (Ikenoya et al., 2002; Sasaki et al., 2002), could alter the magnitude of canonical Wnt signaling by Wnt11, Fz4 and Lrp5 in transiently transfected HEK 293 cells. Interestingly, both inhibitors showed a dose-dependent inhibition of canonical signaling via Fz4 and Lrp5 but not via Fz8 and Lrp5. (B) Increase in Rho-GTP concentration in transiently transfected HEK 293 cells in response to Wnt11 and Fz4 or Fz8. Immunoblots with anti-Rho antibodies showing GTP-bound Rho, captured with a GST-rhotekin fusion protein (upper panel), and total Rho in the postnuclear supernatant (lower panel). (C) Dose-dependent inhibition of canonical Wnt signaling in STF cells by Y-27632 and H-1152P, two inhibitors of Rho kinase (ROCK). Bar charts show mean ± s.d. from triplicate experiments.

**DISCUSSION**

The experiments described here establish an essential role for Fz4 and Fz8 in controlling the size of the developing mammalian kidney. In particular, loss of Fz4 and Fz8 leads to retarded ureteric bud growth and branching, accompanied by decreased expression of Gdnf. The result is a small kidney of apparently normal...
structure. These defects are consistent with a failure of Wnt11 signaling, which can activate canonical and noncanonical signaling via Fz4, but can only activate noncanonical signaling via Fz8 (Fig. 8A).

In genetic mosaics, marked ureteric bud cells lacking Fz4 (in either a Fz4+/– or Fz8–/– background) are substantially under-represented relative to neighboring cells that are heterozygous for Fz4. The magnitude of this proliferative (and/or survival) defect is far greater than one would have predicted based on the phenotype of mice in which Fz4 is missing from all cells. The discrepancy could reflect the compensatory action of other growth regulatory circuits that together act to minimize the proliferative defects that arise from loss of Fz4 signaling (Fig. 8B). In developing kidneys composed entirely of phenotypically WT cells, this model posits that the compensatory growth-promoting signals would be minimally active (Fig. 8B, upper two panels). By contrast, in genetically homogeneous Fz4–/– kidneys, one would expect that compensatory signals would be highly active, although they are insufficient to fully correct the cell proliferation defect (Fig. 8B, lower left). In genetically mosaic kidneys, the compensatory signals would be modestly active, thereby driving an expansion of the pool of phenotypically WT Fz4+/– cells relative to their slow-growing Fz4–/– neighbors (Fig. 8B, lower right).

A potential explanation for the differential abundances of Fz4+/– and Fz4–/– cells in mosaic kidneys is a primary defect in cell behavior that secondarily results in differential cell proliferation, rather than there being intrinsic differences in the rates of proliferation. In fact, within the caudal Wolffian duct there is precedent for a Ret-dependent competitive interaction between neighboring cells that produces both differential cell migration and differential cell proliferation. In chimeric WT:Ret+/– embryos, WT cells preferentially occupy the proliferative domain associated with the primary ureteric bud tip and Ret–/– cells are excluded from it (Shakya et al., 2005; Chi et al., 2009). As a result, in the late-gestation kidney, WT cells dominate the distal branches of the ureteric epithelium and Ret–/– cells are largely confined to the proximal branches. This differential migration and proliferation mechanism could plausibly account for the dramatic difference in the abundance of Fz4+/– versus Fz4–/– (or of Fz4+/–;Fz8–/– versus Fz4+/–;Fz8+/–) cells that we observed in mosaic E12.5 kidneys following Cre-mediated recombination at E8.5. It is unclear whether this mechanism could also account for the differential proliferation of ureteric bud tips in non-mosaic Fz4+/–;Fz8+/– versus Fz4+/–;Fz8–/– kidneys at E15.5 (Fig. 4), a situation in which no competition occurs and the mutant cells occupy all positions within the ureteric bud.

We note that the ‘migration controls proliferation’ mechanism described above is not conceptually at odds with the general feedback model depicted in Fig. 8. If cell proliferation is determined by cell location, then controlling cell migration is one specific mechanism by which cell proliferation could be regulated by a feedback signal.

Regulatory networks for growth control in kidney development

The present work fills in one missing piece in the complex network of cell regulatory signals that coordinate early kidney development. Of particular interest is the prediction that Fz4 and Fz8 loss-of-function mutations decrease the level of Gdnf/Ret/Gfra1 signaling, as judged by the decrease in Gdnf, Ret and Gfra1 transcripts in Fz4+/–;Fz8–/– kidneys. Proliferative defects in both Wnt and RA signaling mutants appear to reflect, at least in part, downregulation of Gdnf signaling. With respect to RA signaling, RARα+/–:RARβ2+/– (Rara+/–:Rarb+/–) mice exhibit a hypoplastic kidney phenotype with downregulation of Ret transcripts. This phenotype can be reversed by forced expression of Ret in the ureteric epithelium (Mendelsohn et al., 1999; Batourina et al., 2001). A similar phenotype is produced by expression of a dominant-negative RA receptor in ureteric buds, which also leads to downregulation of Ret (Rosselot et al., 2010).
Redundancy and robustness in cell signaling pathways

Robustness – the characteristic of resilience in the face of perturbation – is one of the hallmarks of complex biological systems. In vertebrate physiology, homeostatic control of blood pressure, pH and oxygen tension represent classic examples in which tissue health is maintained by multiple feedback loops that adjust respiration rate, heart rate, vascular tone and renal function on a time scale of seconds to hours. Homeostatic mechanisms also operate on time scales of days to years by adjusting red cell production, myocardial and vascular growth and/or renal function, as seen in the chronic responses to hypertension, hypoxia or nephrectomy (Wakatsuki et al., 2004; Ibrahim et al., 2009; Rey and Semenza, 2010). Robustness in the context of embryonic development presents a potentially greater challenge to the organism because compensatory responses must occur rapidly so that different developmental events are not desynchronized.

Genetic redundancy is a well-known strategy for achieving developmental robustness when the perturbation is genetic variation. For diploid organisms, the presence of a second allele confers a minimal level of redundancy. A further level of genetic redundancy can arise from the partially overlapping functions of closely related members of gene families. A more sophisticated strategy for achieving developmental robustness in the face of either genetic or environmental perturbation involves the evolution of signaling systems that refine the rates of cell proliferation, differentiation and survival to match local requirements, where ‘local’ refers to both space and time. Examples include the regulation of embryonic vascular growth by hypoxia inducible factor (HIF) and vascular endothelial growth factor (VEGF) in response to tissue oxygen demand (Rey and Semenza, 2010), and the regulation of neuronal cell death in the dorsal root ganglion by limiting quantities of target-derived trophic signals, such as nerve growth factor (Levi-Montalcini, 1966).

In the present instance, each of the three strategies described above appears to be operating: (1) Fz4 is recessive (allelic compensation); (2) Fz4 and Fz8 are partially redundant (locus compensation); and (3) in the absence of Fz4 and Fz8, the kidney phenotype appears to be partly ameliorated by the compensatory actions of other regulatory systems (pathway compensation). With respect to the third strategy, the genetic mosaic experiments show that failure of Fz4/Fz8 signal transduction in ~50% of ureteric bud epithelial cells has little or no effect on the overall rate of ureteric bud growth and branching and no effect on the postnatal survival of the mice.

A strikingly similar observation has been made in the context of Fgf2 signaling in mouse mammary epithelial cells: when a mixture of genetically marked Fgfr2+/− and Fgfr2−− mammary epithelial cells is implanted into a fat pad, the ducts that develop over the ensuing weeks are composed almost exclusively of Fgfr2−− cells (Lu et al., 2008). It would be of interest to extend this observation by determining whether, in its normal developmental context, a pure population of Fgfr2−− mammary epithelial cells could grow into a mature duct.

Genetic mosaic approaches in mammalian developmental genetics

Genetic mosaic methods in which individual WT and mutant cells are distinguishable by visible markers are rarely used in studies of mammalian development but have been widely used in Drosophila (Simpson, 1976; Wu and Luo, 2006; Potter et al., 2010). Arranging for the coincident deletion (or some other alteration) in the gene of interest and activation of a fluorescent, histochemical or immunohistochemical reporter presents a distinct technical challenge in mammalian systems. In the Drosophila MARCM system, this coincidence is effected by a single Flip-mediated recombination event at the base of a chromosome arm. The small number of Drosophila chromosomes (four), together with the availability of balancer chromosomes, facilitates this approach. Although an analogous approach based on Cre-mediated mitotic recombination has been developed in the mouse (Zong et al., 2005), generalizing it to all chromosome arms will be challenging. Alternative approaches are exemplified by the embryo chimera experiments of Chi et al. (Chi et al., 2009) and by the present work, which is based on a conditional allele that couples deletion of the coding region of interest to the activation of a previously silent reporter gene (Fleischmann et al., 2003; Liu et al., 2008; Badea et al., 2009; Ye et al., 2009). These and other approaches for the activation or inactivation of genes in genetically marked cells should eventually make mosaic analysis a more accessible tool for the study of mammalian developmental biology.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References


Ye et al. Figure S3

The diagram illustrates the genetic manipulation of two transgenic mouse strains, $Fz4^{CKOAP^+/+}$ and $Fz4^{CKOAP^-/-}$, using CreER and 4HT to achieve different genetic outcomes.

- **Fz4^{CKOAP^+/+}**
  - Initially, the gene $Fz4$ is flanked by loxP sites.
  - After CreER + 4HT treatment, the $Fz4$ gene is deleted, and the AP gene is activated.

- **Fz4^{CKOAP^-/-}**
  - Initially, the gene $Fz4$ is flanked by loxP sites, and the AP gene is deleted.
  - After CreER + 4HT treatment, the AP gene is activated, and the $lacZ$ gene is expressed.

This diagram provides a visual representation of the genetic modifications and their outcomes in these two strains.