Stromal–epithelial crosstalk regulates kidney progenitor cell differentiation

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Present models suggest that the fate of the kidney epithelial progenitors is solely regulated by signals from the adjacent ureteric bud. The bud provides signals that regulate the survival, renewal and differentiation of these cells. Recent data suggest that Wnt9b, a ureteric-bud-derived factor, is sufficient for both progenitor cell renewal and differentiation. How the same molecule induces two seemingly contradictory processes is unknown. Here, we show that signals from the stromal fibroblasts cooperate with Wnt9b to promote differentiation of the progenitors. The atypical cadherin Fat4 encodes at least part of this stromal signal. Our data support a model whereby proper kidney size and function is regulated by balancing opposing signals from the ureteric bud and stroma to promote renewal and differentiation of the nephron progenitors.

Kidney development depends on reciprocal interactions between two tissues, the ureteric bud and the metanephric mesenchyme1,2. Signals produced by the mesenchyme promote reiterative branching morphogenesis of the ureteric bud, and signals from the bud support survival and proliferation of nephron progenitor cells within the mesenchyme. In addition, the bud produces a signal(s) that induces a subset of the progenitors to undergo a mesenchymal-to-epithelial (MET) transition to form an intermediate condensed structure known as the pre-tubular aggregate (PTA), which proceeds to an epithelial structure referred as the renal vesicle. The renal vesicle will undergo morphogenesis to form the nephron, a structure consisting of the renal corpuscle, the proximal tubule, the loop of Henle, the distal tubule and the connecting tubule. The ureteric bud will give rise to the collecting ducts and ureter.

A significant portion of the inductive activity attributed to the ureteric bud can be assigned to Wnt9b (ref. 3). Previous studies have shown that Wnt9b signals to the nephron progenitor cells and activates at least two molecularly and spatially distinct programs; one that promotes progenitor cell proliferation/renewal (referred to as the Class II/progenitor signature) and another that induces their differentiation (referred to as the Class I/pre-tubular aggregate or PTA signature)4. In the absence of Wnt9b, the progenitor domain is specified correctly but does not expand and the PTAs and renal vesicles do not form3,4. Both programs are activated by the transcription factor β-catenin4. A question that arises is how the same molecule promotes two seemingly contradictory, program-specific responses.

The nephron progenitors are encapsulated by a population of fibroblasts known as the stroma (Fig. 1a). These cells are ideally positioned to influence the fate of the nephron progenitors. Indeed, ablation of the transcription factor Foxd1 from the stroma results in expansion of nephron progenitor cells and a severe deficit in MET/differentiation3,6. However, the precise mechanism underlying this phenotype is unclear.

Here, we show that the stromal cells produce a signal(s) that regulates progenitor cell renewal. This signal is at least in part encoded by the atypical cadherin Fat4. Fat4 normally functions to modify β-catenin activity, promoting the differentiation program and repressing the renewal program. We propose that Fat4 accomplishes this role by modulating the activity of Yap and Taz within the nephron progenitors. By providing opposing progenitor renewal and differentiation signals, the ureteric bud and the stroma provide a niche that ensures proper nephron endowment and optimal kidney function.

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Received 8 December 2012; accepted 16 July 2013; published online 25 August 2013; corrected online 29 August 2013; DOI: 10.1038/ncb2828
**RESULTS**

**The stroma promotes differentiation**

To test a potential role for the stroma in nephron progenitor fate, these cells were ablated by generating pups carrying both a Cre inducible form of the diphtheria toxin A chain (RosaDTA; ref. 7) and Foxd1Cre (ref. 8). Foxd1Cre;RosaDTA pups died within 24 h of birth. As expected, examination of embryonic day (E)18.5 kidneys revealed a complete absence of the cortical stromal cells and their derivatives (Supplementary Fig. S1A). Foxd1-positive stromal cells were absent at E15.5 although there were still some Foxd1-derived medullary stromal cells (Supplementary Fig. S1A). Thus, the Foxd1Cre;RosaDTA mouse results in deletion of the cortical stroma by at least E15.5.

Kidneys of P1 Foxd1Cre;RosaDTA pups (which we will refer to as stromaless) were smaller than wild-type kidneys and were fused to the body wall. Haematoxylin–eosin-stained sections revealed an expanded zone of mesenchymal cells capping the ureteric buds (Fig. 1c). Staining with an antibody against Six2 demonstrated that the nephron progenitor domain of mutants was significantly expanded in the Foxd1cre;Rosa26DTA mutants (Fig. 1e, g, i).

To determine whether stromaless kidneys had a normal Wnt9b response, we assessed the expression of both Class I/PTA and Class II/progenitor target genes at E15.5. Class I targets include Pax8, Wnt4, Lef1 and Cldc2 (ref. 4). Class II targets include Cited1, phospholipase A2 group 7 (Pla2g7), Amphiphysin (Amph) and expressed sequence AW049604 (Tafa5/Faml9a5; ref. 4). At E15.5, all Class II/progenitor Wnt9b targets examined were expressed throughout the expanded progenitor domain of the stromaless mutants (Fig. 1e, g and Supplementary Fig. S1B). However, the expression of Class I/PTA targets was significantly reduced or absent (Fig. 1i and Supplementary Fig. S1B). Although most kidneys were largely devoid of PTAs and renal vesicles, a very low number of PTAs and renal vesicles did form, most likely corresponding to regions of retained stroma. These data suggest that a signal(s) from the cortical stroma suppresses renewal and/or promotes differentiation of the nephron progenitor cells.

Previous studies have found that the stroma produces secreted frizzleds and it has been suggested that these signals affect the activity of Wnt ligands produced by the ureteric bud and/or renal
vesicles. To determine whether ablation of the stroma could affect the strength of ureteric-bud-derived Wnt9b signalling, we cultured stromaless mutants with a small molecule that inhibits Wnt production (IWP2) by repressing the fatty acyl transferase.

Unexpectedly, Foxd1cre;RosaDTA kidneys treated with the highest dosage of IWP2 still maintained the expression of Wnt9b Class II/progenitor targets (although at slightly reduced levels) after 48 h of culture (Fig. 2c). Interestingly, IWP2 treatment blocked Wnt production (Fig. 2d). Ablation of stromaless kidneys led to loss of Wnt9b Class II target expression (Supplementary Fig. S6). These data suggest that in the absence of stroma, Class II target expression is less dependent on a Wnt ligand, but does not lessen dependence on β-catenin.

To determine whether β-catenin was still necessary for the expression of Wnt9b targets in stromaless kidneys, Foxd1cre;RosaDTA kidneys were cultured with a compound that destabilizes β-catenin (inhibitor of Wnt production; b,c,h) or the β-catenin destabilizer IWR1 (c,f,i). All tissues were hybridized with an antisense probe against Pla2g7. Scale bars, 1 mm. (j,k) Quantitative analysis of gene expression by quantitative PCR with reverse transcription (qRT–PCR). cDNA levels of Class II/progenitor targets Tafa5 and Pla2g7 and Class I/PTA target C1qdc2 were assessed after treating wild-type or Fat4 null kidneys with IWP2 (j) or IWR1 (k) or dimethylsulphoxide. Dimethylsulphoxide-treated wild-type levels are considered baseline. Shown is the mean value of qRT–PCR data from three different experiments. Error bars indicate s.e.m. *P < 0.05; #P < 0.01.

**Figure 2 Expression of Class II targets is independent of a Wnt ligand but dependent on β-catenin in stromaless and Fat4 null mutants.** (a–i) Whole-mount images of wild-type (a–c), Foxd1cre;RosaDTA (d–f) and Fat4 null (g–i) kidneys cultured from E11.5 for 48 h in the presence of dimethylsulphoxide (DMSO; a,d,g), 5 μM IWP2 (inhibitor of Wnt production; b,e,h) or the β-catenin destabilizer IWR1 (c,f,i). All tissues were hybridized with an antisense probe against Pla2g7. Scale bars, 1 mm.
Figure 3 Nephron progenitors of stromal mutants show increased nuclear Yap and reduced pYap. (a–l) P1 wild-type (a–d), Foxd1cre;Rosa26DTA (e–h) and Fat4 null (i–l) kidneys were subjected to immunostaining using either an antibody against Yap or a pYap-specific antibody (green in merged images), the progenitor marker Six2 (in red) and the ureteric bud epithelium marker cytokeratin (CK; in blue). Single channel images for pYap (b, f and j) and Yap (d, h and l) are shown beneath each merged image. The asterisks indicate the progenitor domain in mutants with significantly reduced pYap expression. All images were captured at ×63 magnification. Scale bars, 20 μm.

Yap protein levels were next assessed in Foxd1Cre;RosaDTA kidneys. Although total Yap levels did not change significantly in mutants, there was a striking increase in the levels of nuclear Yap in all three lineages within the nephrogenic zone of mutant kidneys (Fig. 3g, h). Conversely, phosphorylation of Yap was significantly reduced in the Six2-positive cells of Foxd1Cre;RosaDTA kidneys (Fig. 3e, f). These data suggest that signals from the cortical stroma regulate Yap phosphorylation and localization within the nephron progenitors.

Yap/Taz regulates Wnt9b activity

Our data suggest that nuclear Yap (and Taz) stimulate the expression of Wnt9b target genes. To test this hypothesis, nephron progenitors were isolated and cultured. As in the stromaless mice, Yap protein was predominantly nuclear in the cultured cells although, as previously shown, the levels of nuclear Yap were inversely correlated to cell density (Supplementary Fig. S2B).

Cited1, Pla2g7, Tafa5 and Amphiphysin are all expressed in the isolated progenitors in the absence of Wnt9b (Fig. 4a) and all tested were positively affected by Lef1/β-catenin activity (Supplementary Fig. S4A). We next investigated whether the expression of these genes was dependent on Wnt production. Consistent with observations made in Foxd1Cre;RosaDTA kidneys, treatment of isolated progenitor cells with IWP2 had no significant effect on the expression of these progenitor target genes (Supplementary Fig. S4B).

We next investigated whether Wnt9b/Class II target expression was dependent on Yap/Taz activity. Cells in which Yap/Taz mRNA/protein levels were knocked-down using short interfering RNA (siRNA)-mediated silencing showed a significant repression of the Class II/progenitor targets Cited1, Tafa5, Pla2g7 and Amphiphysin (Fig. 4a, b) whereas the levels of Wnt9b-independent progenitor targets such as Sall1 and Six2 were only moderately reduced (Fig. 4a, b). Expression of the class I/PTA targets Pax8 and E-cadherin was...
significantly increased in these cells (Fig. 4a,b). These findings suggest that nuclear Yap/Taz enhances the expression of Wnt9b Class II/progenitor target genes whereas it represses the expression of Class I/PTA targets.

To determine whether this role for Yap/Taz was conserved in vivo, we ablated both genes from the kidney nephron progenitors using Six2Cre. Both Yap and Taz are expressed in largely overlapping domains in the kidney progenitor cells (Supplementary Fig. S8B).
Figure 5 Abolition of Yap/Taz from the progenitors alters expression of Class I and Class II target genes in the nephron progenitors. (a–i) Sections from E18.5 wild type (a,c,e,g,i,k,m,o,q,q′,q'') or Six2Cre; Yap^{floxed}/Taz^{floxed} mutant (b,d,f,h,j,l,n,p,r) kidneys were stained with antibodies against pYap (green), the progenitor marker Six2 (red) and the ureteric bud epithelium marker dolichos biorus agglutinin (DBA, in red) and DAPI (blue; a,b; single-channel images for pYap are shown in a′,b′), haematoxylin and eosin (H&E; c,d); sections were stained with antibodies against LTL (red), the epithelial marker E-cadherin (green) and DAPI (blue; e,f), the Wnt9b/Class II target Cited1 (in green), the ureteric bud epithelium marker dolichos biflorus agglutinin (DBA, in red) and DAPI (blue; g,h; g′,h′ show a higher magnification with the ureteric bud outlined in white), the antisense Wnt9b/Class II target Pla2g7 (i,j), the antisense Wnt9b/Class II target Tafa5 (k,l), the antisense Wnt9b/Class II target Pax8 (m,n; m′,n′ show the higher magnification with the ureteric bud outlined in white), the antisense Wnt9b/Class I target Wnt4 (o,p), antibodies against the Class II target Amphiphysin (in red), β-catenin (in green) and E-cadherin (blue; q,r), the corresponding single channels for Amphiphysin and β-catenin are shown in q′,r′ and q″,r″, respectively. (s,t) Model showing the expression of progenitors in wild type and Yap/Taz double mutants. The self-renewing progenitor cells (light grey) are significantly reduced or lost in the mutants and are replaced by the differentiating progenitors (dark grey). Dashed lines indicate the ureteric bud in all panels. Arrows in a,b and a′,b′ indicate reduced Six2-positive cells and the asterisks indicate the PTAs; arrows in c,d indicate epithelial structures lost in the mutants; arrow in h indicates loss of Cited1 expression from ureteric bud tips; and arrows in n and p indicate ectopic expression of target genes. Scale bars, 0.1 mm (a,b), 10 μm (c–p) and 50 μm (q,r).

and single ablation of either gene had little effect on Wnt9b target gene expression (with the exception of Cited1, which was lost in both single mutants Supplementary Fig. 8C). Although Six2Cre; Yap^{floxed}/Taz^{floxed} mutants maintained Yap protein to E13.5, levels were greatly decreased at E15.5 and by birth, most of the progenitor cells and their derivatives showed no detectable levels of Yap (Supplementary Fig. S3 and Fig. 5a,b). As expected, given the kinetics of Yap ablation, the first several days of kidney development did not seem to be affected in these mutants (Supplementary Fig. S3). However, Six2Cre; Yap^{floxed}/Taz^{floxed} mutants died within 24 h of birth.

At E18.5, Yap/Taz double mutant kidneys were smaller than those of wild-type littermates and exhibited a greatly reduced nephrogenic zone and decreased density of epithelia in the more central regions of the kidney indicative of a reduction in nephron number (Fig. 5d). Staining of mutant kidneys with Lotus tetragonolobus lectin (LTL), a marker of proximal tubules, revealed a severe decrease in the number and length of these structures in mutants (Fig. 5e,f). Further, P1 double mutants had significantly fewer glomeruli than wild-type kidneys (Supplementary Table S1). However, the collecting duct epithelium, which is derived from the ureteric bud lineage, looked relatively normal.

The paucity in progenitor-cell-derived structures in mutants could be caused by a deficit in progenitor cell number or progenitor cell
**Fat4 regulates Yap/Taz activity in the progenitors**

Our data suggest that a signal(s) from the stroma regulates Yap/Taz activity within the progenitors. In flies, members of the Fat and Ds family of atypical cadherins have been shown to mediate the activity of the Yap/Taz homologue Yorkie. Intriguingly, one member of this family, Fat4, is expressed predominantly in the stroma of the embryonic kidney.

To determine whether Fat4 regulates the phosphorylation status of Yap/Taz, we analysed the expression of the Yap protein in embryonic kidneys. Consistent with the observations in the stromaless kidneys, phosphorylation of Yap was normal in the stroma and the ureteric bud epithelium, but significantly reduced in most Six2-positive progenitor cells in Fat4 mutant kidneys. Further, total Yap protein was predominantly localized in the nucleus of Fat4 mutant progenitor cells. These results suggest that Fat4 regulates the activity/localization of Yap in the embryonic kidney.

**Fat4 nulls exhibit an expanded progenitor domain**

We next sought to determine whether ablation of Fat4 had an impact on the nephron progenitor population. Fat4 null kidneys are smaller than their wild-type littermates at birth but seem to have an expanded progenitor domain. The expanded progenitor cells expressed Wnt9b nephron progenitor (Class II) targets as well as other progenitor markers. Although we observed ectopic expression of Class I/PTA targets, the formation of renal vesicles seemed to be delayed in mutants suggesting that Yap/Taz activity is required for proper differentiation. Thus, the reduced size of the kidney and the reduction of nephron number observed in Fat4/Taz mutants are most likely caused by a combination of reduced progenitor cell renewal as well as defects in differentiation.

**Figure 6** Fat4 nulls exhibit an expanded progenitor domain and reduced differentiation. (a–p) Wild-type (a,c,e,g,i,k,m,o) and Fat4 null (b,d,f,h,j,l,n,p) E18.5 kidneys stained with haematoxylin and eosin staining (a,b), antisense probes against Six2 (c,d) and Eya1 (e,f) and the Wnt9b/Class II target genes Cited1 (g,h) and Taf5 (i,j), or antibodies against Amphiphysin (green), the ureteric bud epithelium marker cytokeratin (CK in red) and DAPI (k,l), Pax2 (red), E-cadherin (green) and β-catenin (blue; m,n), Six2 (red), the Wnt9b/Class I target gene Lef1 (green) and cytokeratin (blue; o,p). Quantification of Six2-positive cells revealed that the progenitor domain was on average 4 cell layers thick in mutants as compared with 2.1 cell layers in wild-type kidneys (n = 4, P < 0.000001). Wild-type kidneys had an average of 51.5 Lef1-positive structures per section whereas Fat4 mutants had only 28 (n = 4, P = 0.007). Statistics source data can be found in Supplementary Table S1. All images are taken at ×20 magnification. Scale bars, 100 μm.
Figure 7 Fat4 mutants express Class II/progenitor genes in the absence of Wnt9b. (a-x) Wild-type (a,e,i,m,q,u,a’), Fat4 mutant (b,f,j,n,r,v,b’), Wnt9b mutant (c,g,k,o,s,w,c’) and Wnt9b/Fat4 double mutant (d,h,l,p,t,x,d’) kidneys at P1 (a-d and a’-d’), E11.5 (e-t) or E15.5 (u-x). Sections were stained with haematoxylin and eosin (a-d), anti-Six2 (green), DBA (red) and DAPI (blue; a’-d’), anti-Six2 (red), the Wnt9b/Class II target gene Cited1 (green) and cytokeratin (blue, CK in e-h), antisense probes against Pla2g7 (i-l), Tafa5 (m-p) and the Wnt9b/Class I target genes Clgdc2 (q-t) and Wnt4 (u-x). Dashed lines indicate the ureteric bud. The asterisks in a-d indicate expanded progenitor cell domains. The asterisks in w-x indicate medullary stromal expression of Wnt4. The letter a in w,x indicates the adrenal gland. In a-d images are captured at ×40 magnification and scale bars, 20 μm. All other images are at ×20 magnification and scale bars are 100 μm.

We next determined whether Fat4 mutant kidneys continued to express Wnt9b/Class II target genes after treatment with IWP2 (similar to stromaless kidneys). Indeed, the low level expression of Wnt9b Class II target genes was maintained, whereas Class I/PTA targets were lost (Fig. 2h,j,k and Supplementary Fig. S6). Fat4 mutants lost expression of both classes of targets after treatment with IWR1 (Fig. 2l,j,k and Supplementary Fig. S6). Thus, ablation of Fat4 renders expression of Wnt9b Class II/progenitor targets less dependent on a Wnt ligand but does not rely on dependences on β-catenin.

To determine the effect of Fat4 ablation on Wnt9b activity in vivo, we generated Fat4/Wnt9b double mutants. Fat4/Wnt9b double mutant kidneys were hypoplastic with a moderately branched ureteric bud system. Unlike Wnt9b null kidneys, double mutant buds were capped with progenitor cells although they still lacked renal vesicles and their derivatives (Fig. 7d). Consistent with the results observed on culture of Fat4 mutants with IWP2, we found that expression of Wnt9b Class II/progenitor target genes Cited1, Pla2g7 and Tafa5 was rescued in Wnt9b mutants on co-ablation of Fat4 (Fig. 7h,l and p). However, class I/PTA target expression was not rescued (Fig. 7t,x). These data suggest that Fat4 inhibits the expression of Wnt9b Class II/progenitor targets and promotes the expression of Class I/PTA targets in vivo.

Previous studies suggest that Fat4 interacts with Vangl2 to regulate PCP during tubule diameter maintenance in the kidney26. However, removal of Vangl2 from mice carrying either a null or hypomorphic allele of Wnt9b (ref. 27; Wnt9b+/–; Vangl2Δlp/lp and Wnt9bΔneo/neo; Vangl2Δlp/lp) did not rescue the progenitor domain or expression of Wnt9b Class II/progenitor targets, suggesting that this phenotype is independent of the Vangl2/PCP pathway (Supplementary Fig. S7B).

The cortical stroma and mural cells of Fat4 mutant kidneys were molecularly and phenotypically indistinguishable from the wild type (Supplementary Fig. S7A). Further, we did not observe ectopic expression of progenitor markers within the stromal compartment of Fat4 mutants (Supplementary Fig. S7A). These data suggest that the expansion in progenitors was not caused by a transformation of the stroma into progenitors.

Reciprocally, lineage tracing of progenitor cells in kidneys revealed that the Six2Cre-expressing derivatives were present in Wnt9b mutants until E15.5 (Supplementary Fig. S8A). Co-labelling of Six2Cre;RosaYFP; Wnt9bΔlp/lp with antibodies against GFP and stromal markers Foxd1, Meis1/2 or Slug demonstrated that the progenitors of Wnt9b mutants were not mis-specified or trans-fated towards a stromal fate (Supplementary Fig. S8A). These findings rule out the possibility that the phenotypes observed in Wnt9b, Fat4 or Wnt9b/Fat4 double mutants were the results of defects in nephron progenitor or stromal cell specification or fate.

Fat4 acts non-autonomously

Our model suggests that stromal Fat4 acts on the adjacent progenitors. To determine whether Fat4 can act non-autonomously, we co-cultured isolated progenitors with cells transfected with full-length Fat4. Yap localization shifted from predominantly nuclear to cytoplasmic when progenitor cells were cultured adjacent to Fat4-expressing cells (Fig. 4d). A construct lacking the cytoplasmic domain of Fat4 (Fat4-ECD) had the same effect (Fig. 4c). Both constructs resulted in increased levels of phosphorylated Yap. All results were independent
of cell density, indicating that Fat4 is capable of activating Yap/Taz in a non-autonomous manner.

We next assessed the effect of Fat4 on progenitor cell differentiation. Both full-length and the extra-cellular domain of Fat4 had a similar effect on gene expression as knockdown of Yap/Taz, repressing class II/progenitor gene expression (with the exception of Pla2g7) and enhancing differentiation/MET as assessed by E-cadherin (Fig. 4c). In sum, these data suggest that stromally derived Fat4 non-autonomously regulates Yap/Taz activity within a subset of the nephron progenitors, which promotes their differentiation.

DISCUSSION

The embryological studies performed by Clifford Grobstein during the 1950s established one of the central tenets of metanephric kidney development: nephron progenitor maintenance and differentiation rely on signals produced by the ureteric bud epithelium. However, there is growing evidence that final kidney form relies on inductive and inhibitory crosstalk between multiple cell types present in the organ anlagen. In this study, we provide evidence that the cortical stroma inhibits nephron progenitor cell expansion and promotes its differentiation. We propose that Fat4, produced by the stroma, provides a signal that antagonizes nephron progenitor renewal and promotes differentiation by modulating response to Wnt9b.

We suggest that Yap/Taz plays a crucial role in regulating Wnt9b signature activation. Precisely how this is accomplished is not clear. Several recent studies have shown direct interaction between β-catenin and Yap/Taz signalling. Our data are consistent with the idea that Fat4 signalling promotes the expression of Wnt9b/β-catenin differentiation targets, and inhibits the expression of the progenitor renewal targets. We propose that nuclear Yap/Taz and β-catenin cooperate to activate the nephron progenitor signature and loss of nuclear Yap/Taz (and possibly gain of cytoplasmic pYap/Taz) is necessary for the expression of the differentiation signature (Fig. 8). As we have not identified conserved Tead- (the DNA binding co-factor for Yap and Taz) binding sites within either class of target gene, the effect of Yap/Taz on β-catenin targets may not be direct. Nevertheless, Yap/Taz must impinge on Wnt9b/β-catenin activity at some point.
Although Fat4 is expressed in the stroma (refs 24,25 and this study), we cannot rule out the possibility that low levels of Fat4 present in the progenitors constitute the active pool of protein. However, the observation that DTA deletion of the stromal cells results in a similar phenotype to Fat4 ablation would seem to support a stromal source. We propose that Fat4 binds to another factor produced by the progenitors to activate Yap/Taz in the progenitors. The mouse orthologue of Dachsous and Fat3, a paralogue of Fat4, are both expressed in the progenitor cells where they could be acting as receptors for stromal Fat4 (refs 24,25,31). It will be interesting to determine whether either of these factors mediates Yap/Taz activity.

The Foxd1Cre;Rosa26DTA kidneys elicit a more severe phenotype than mutation of Fat4 alone. Inappropriate production of other signals (such as Bmps, ref. 6) produced by the ectopic endothelial or capsular cells observed in Foxd1 (ref. 6) mutants may lead to the more severe phenotype observed in these mutants. In support of this model, we did not detect ectopic pSmad1/5/8 activity in Fat4 or SixCre; Yapflx/fox, Tazflx/fox mutants (Supplementary Fig. S7C), although they are detectable in Foxd1 mutants.6 These data suggest that the phenotypes resulting from ablation of Fat4 are a subset of those observed in Foxd1 and stromalless kidneys.

In summary, we have revealed that ureteric-bud-derived Wnt9b and stromal-derived Fat4 provide opposing signals that regulate kidney progenitor cell maintenance and differentiation. Although this interaction is clearly essential for kidney development, we feel that the crosstalk between the Wnt and Fat/Yap pathways may be representative of a more general mechanism underlying stromal/epithelial interactions in multiple tissues. Given the increasing evidence of stromal involvement in numerous human pathological conditions, these findings are likely to have a significant impact on our understanding of human disease.

METHODS

Methods and any associated references are available in the online version of the paper.

ACKNOWLEDGEMENTS

We thank Y. Yang (NIH, USA) for providing the Vangl2 mutant mice, M. Takeichi (RIKEN Center for Developmental Biology, Japan) for providing the Fat4 full-length reagents. The paper was written by A.D., S.T., A.O.P. and T.J.C.

AUTHOR CONTRIBUTIONS

Experiments were designed by A.D., S.T., C.M.K., A.O.P. and T.J.C. Experiments were performed by A.D., S.T. and C.M.K. Data were interpreted by A.D., S.T., C.M.K., A.O.P. and T.J.C. M.X., L.L., C.C. and E.N.O. provided mice or other reagents. The paper was written by A.D., S.T., A.O.P. and T.J.C.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Note: Supplementary Information is available in the online version of the paper.

Published online at www.nature.com/doifinder/10.1038/ncb2828
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METHODS

Mice. All animals were housed, maintained, and used according to protocols approved by the Institutional Animal Care and Use Committees at the University of Texas Southwestern Medical Center and following the guidelines from the NCI-Frederick animal care guidelines. Four-week-old female mice of 7–8 weeks of age were crossed with a male of 9–10 weeks of age. Plugs were checked and the embryos were collected at the desired time points for further analysis. The number of mice used for each experiment was as follows: Wnt9bwt+/− (5–6 pregnant females at each time point), Wnt9b(wt/−) (5 pregnant females), Fat4−/− (6–8 pregnant females for each time point), Rosa26DTA (6–8 pregnant females for each time point and 6–7 to collect E18.5 and P1), Yang2 and Ror (2–3 pregnant females for each), Six2 Cre, Foxd1Cre and KspCre (1 male for each). Mice of the desired genotype were randomly selected and the investigator was blinded to allocation.

Creation of a conditional Yap and Taz mutant allele. The Yap and Taz targeting vector was constructed using pKneoF2L2DTA harbouring two loxP sites encompassing a neomycin resistance cassette flanked by two FRT sites. Targeting arm sequences were isolated from 129SvE genomic DNA by PCR. The Yap 5′ targeting arm (5 kb), Yap 3′ targeting arm (4 kb), Yap knockout arm (2 kb), Taz 5′ targeting arm (5 kb), Taz 3′ targeting arm (4 kb) and Taz knockout arm (2 kb) were cloned into the vector using NotI, Xmal, EcoRV, EcoRV, EcoRV and Xmal respectively. The sequence-verified targeting vector was linearized and electroporated into 129SvE-derived embryonic stem cells. Targeting of the mutant allele was screened through Southern blot analysis. The Yap 5′ probe detects a 6.3 kb DNA fragment in addition to the wild-type 9 kb fragment after EcoRI digestion and the Yap 3′ probe detects a 10 kb DNA fragment plus the wild-type 8 kb fragment after PstI digestion in the presence of the Yap-targeted allele. Similarly, the Taz 5′ probe detects a 10 kb fragment in addition to the wild-type 12 kb fragment on StuI digestion and the Taz 3′ probe detects an 8 kb fragment in addition to the wild-type 10 kb fragment on BamHI digestion if homologous recombination occurred. Targeted ES cells were injected into blastocysts to generate chimaeric mice. High-percentage chimaeric males were bred with mice expressing FLPe recombinase to remove the neomycin resistance cassette, producing Yaplox/− and Tazlox/−. PCR genotyping using a forward primer upstream of the 5′ loxP site, a first reverse primer located in the knockout arm, and a second reverse primer downstream of the 3′ loxP site produces 600 base pair (bp), 437 bp, 338 bp, 655 bp, 496 bp and 704 bp bands for Yap−/−, Yap+/−, Yaplox/−, Taz−/−, Tazlox/− and Tazlox−/− alleles, respectively. Primer sequences for PCR genotyping are as follows: YF: 5′-ACATGATGCTGGGCGGAG-3′; YR1, 5′-AGGCTGAGACAGGAGGATCTCTGTGAG-3′; YR2, 5′-TGGTGAAGACGTCGTATTACGAGGCT-3′; TF, 5′-GGCTTGAGACAAAAAGTGGGGCTATCGT-3′; TR1, 5′-CCACTAGTTATAGCTGTCCCAAGACTGGG-3′; TR2, 5′-AAGCCTGAGGAGGACCTGCTCCGTCGAC-3′.

Ex vivo organ culture. Isolated kidney explants were cultured as previously described. Briefly, E11.5 kidneys were cultured at the air/medium interface for 48 h. Small-molecule Wnt antagonists (5 μM of IWP2 and 100 μM of IWR1) were added to the medium and replaced every 12 h. After 48 h of culture the kidneys were fixed in ice-cold 4% paraformaldehyde and washed thoroughly in PBS before subjecting them to further analysis. All analyses were repeated at least three times, with a minimum of four different kidneys for each analysis.

Tissues and primary metanephric mesenchyme cell cultures. Metanephric mesenchymes were enzymatically separated from the T-shaped ureteric buds from E13.5 rat embryos and cultured on laminin-coated BD-Biocat 6-cm dishes (BD Biosciences) in serum-free Ham’s F12/DMEM: 1 (Gibco, BRL) medium with supplements as described previously (ref. 21) and with the addition of 10 ng ml−1 human TGF-α and 50 ng ml−1 human FGF2. Tissues were grown for 10 days, resulting in a 50–100-fold increase in cell numbers. Cells were removed from plates by trypsin–EDTA solution (Gibco–Invitrogen).

Plasmids and electroporation. Lef-1/β-catenin fusion complementary DNA was generously provided by W. Bichmeier and J. L. Gordon (ref. 3), and cloned into pCMV–tag3–myc plasmid (Stratagene). Primary metanephric mesenchyme cells were transfected with the Amaxa Nucleofector 96-well Shuttle System (Lonza Group). Briefly, 1 million cells per transfection were resuspended in 20 μl of P3 Primary Cell 96-well Nucleofector Kit (Lonza). pCMV–tag3–myc or pCMV–tag3–myc–Lef-1/β-catenin plasmid (2 μg) was mixed with cell suspensions and transferred to the well of a 96-well Nucleoplast plate. For Fat4 experiments, empty vector or Fat4 plasmids (4 μg) were mixed with 1 million cells in 20 μl of 96-well Nucleofector. Nucleofector conditions were performed according to the manufacturer’s instructions using program DN-100, and then cells were seeded on laminin-coated 24-well. At 24–72 h post transfection, cells were collected by TRIZOL (Invitrogen) for RNA isolation or RIPA buffer for protein analysis.

siRNA transfection. Scrambled siRNA (#4390843, Ambion), Yap siRNA (sense: 5′-GGUUAGAUCAAGUAAAACATT-3′, anti-sense: 5′-AUUGGUUUAGUAGUAAACT-3′, #4390771, siRNA ID: si170198, Ambion) or ON-TARGETplus SMART pool Rat Wtwt1 (Taz) siRNA (1: #5-GAGAUAGCCUACCCGGCA-3′; 2: #5-AGCUCAUAUGGAGUCA-3′; 3: #5-CUAAAGUUGUAAACAAAU-3′; #4: 5′-GGGUAAAUUCCGGUCAGA-3′, siRNA ID: L-088521-02-0005, Dharmacon) was transfected into rat metanephric mesenchyme cells by Lipofectamine RNAi MAX (Invitrogen) transfection reagent following the manufacturer’s protocol. Culture medium was changed 24 h post transfection and transfection efficiency was analysed by qRT–PCR or immunoblotting.

In situ hybridization. For section in situ hybridization, kidneys isolated at specific stages were fixed overnight in 4% PFA (in PBS) at 4°C and cryopreserved in 30% sucrose. Tissues were frozen in OCT (Tissue Tek) and sectioned at 10 μm. Sections were subjected to in situ hybridization as previously described. The following probes were used: Fat4 (linearized with BamHI and transcribed with T3 polymerase), Yap (linearized with BamHI and transcribed with T3 polymerase), Taz (linearized with EcoRI and transcribed with T3 polymerase), Cited, Six2, Sall Plaq27, Cldc2 and Wnt4 (ref. 4).

Histology, immunohistochemistry and immunocytochemistry. Kidneys isolated at birth were formaldehyde fixed and paraffin embedded. Sections (5 μm) from paraffin-embedded kidneys were subjected to haematoxylin and eosin staining. For immunohistochemistry, fixed kidneys were embedded in OCT and sectioned on a cryostat. Frozen sections were manually coated with laminin and stained with 5% human FGF2. Tissues were grown for 10 days, 1 μg ml−1 human Lef-1/β-catenin fusion complementary DNA was added to the medium and replaced every 12 h. After 48 h of culture the kidneys were fixed in ice-cold 4% paraformaldehyde and washed thoroughly in PBS before subjecting them to further analysis. All analyses were repeated at least three times, with a minimum of four different kidneys for each analysis.

Immunocytochemistry. Primary metanephric mesenchyme cells were cultured (low density: 0.1 million, high density: 1 million cells) on 4-well chamber slides (Thermo, #153382), which were manually coated with laminin (5 μg per well, BD Biosciences). Cells were washed with PBS, fixed in 4% paraformaldehyde for 10 min and further washed 3 times with PBS, 5 min each. The cells were then blocked for 1 h at room temperature with 10% goat serum in PBS, and incubated with monoclonal antibodies (1:50) overnight. The cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated secondary antibody for 1 h. The cells were treated with DAPI, mounted on slides with Vectashield and images were captured with a Zeiss LSM510 microscope. For pSmad1/5 stains, frozen sections were fixed with 4% PFA, permeabilized with 1% SDS at 37°C for 10 min, blocked for an hour at room temperature (with 1% BSA + 0.1% fish gelatin + 0.1% Tween 20) and stained with polyclonal antibodies. Slides were incubated overnight with primary antibodies. After primary incubation, sections were washed and incubated with HRP-tagged secondary antibodies for 1 h at room temperature. Further, signal was detected with tyramide amplification. Slides were washed and re-stained with additional markers according to the above-mentioned immunohistochemistry protocol. Slides were then mounted with Vectashield and images were captured with a Zeiss LSM510 confocal microscope. For pSmad1/5 stains, frozen sections were fixed with 4% PFA, permeabilized with 1% SDS at 37°C for 10 min, blocked for an hour at room temperature (with 1% BSA + 0.1% fish gelatin + 0.1% Tween 20) and stained with polyclonal antibodies (Cell Signaling, Cat. 9511, 4121; 1,500, tyramide amplified) and pY (Cell Signalling 1,500, tyramide amplified) antibodies, washes were carried out in 1× PBS with 0.1% Tween 20 instead of PBS, and sections were antigen retrieved using citrate buffer at pH 6.0 and blocked with 10% normal goat serum. Slides were incubated overnight with primary antibodies. After primary incubation, sections were washed and incubated with HRP-tagged secondary antibodies for 1 h at room temperature. Further, signal was detected with tyramide amplification. Slides were washed and re-stained with additional markers according to the above-mentioned immunohistochemistry protocol. Slides were then mounted with Vectashield and images were captured with a Zeiss LSM510 confocal microscope.
cocktail (Roche). Cell lysates were homogenized in an ultrasonicator for 15 s four times on ice. The protein concentration was determined by the Rc-protein assay kit with a BSA standard (Bio-Rad Laboratories). Protein samples (10 μg each) were denatured by 4x LDS buffer at 75 °C for 10 min and resolved in 4-2% Bis-Tris gels with MOPS buffer (Invitrogen). Proteins were electrotransferred to PVDF membranes (Bio-Rad, Hercules). Membranes were blocked with 5% non-fat dry milk in TBST and incubated overnight at 4 °C with primary antibodies. Membranes were washed with TBST, followed by species-specific HRP-conjugated secondary antibodies. Bound antibodies were visualized using the SuperSignal West Pico Chemiluminescent Substrate system (ThermoFisher Scientific) according to the manufacturer’s instructions. Antibodies were obtained as follows: against Yap/Taz (#8418), pYap (#4911), Yap (#4912), phospho-MST (#3681) from Cell Signaling Technology; against CITED1 (#RB-9219) from Lab Vision; against CAG (#15526) and Amphiphysin #610181) from BD Biosciences; against CITED1 (#RB-9219) from Lab Vision; against Sall1 (#b31526) from Abcam; against Taf4a5 (#F5148) from R&D systems; Yap monoclonal antibody (M01; #H00010413) from Abnova.

**In vitro co-culture.** To distinguish between the Fat4-expressing effector cells and the wild-type progenitor cells, we cultured cells on laminin-coated 4-well coverslips after co-transfection of Fat4 plasmids (4 μg per 1 million cells) and GFP plasmids (0.5 μg per 1 million cells) by electroporation as described above. Twenty-four hours later wild-type metanephric mesenchyme cells (progenitor cells stained with CellTracker Red CMTPX, Invitrogen) were added to the effector cells (0.5 million cells per well). Cultured metanephric mesenchyme cells were fixed with 4% PFA for immunofluorescence microscopy after 24 h culture as described above.

**Progenitor cell layer counts.** Kidneys were sectioned and stained for ureteric bud (cytokeratin or DBA) and progenitor cell markers (Cited1 and/or Six2). Sections that passed through the lumen of the ureteric bud were subsequently used to count progenitor cells. The progenitor cell layer atop the ureteric bud was counted from 3--4 different kidneys and an average was calculated per ureteric bud tip for each kidney.

**Glomerular counts.** Kidneys isolated at birth were paraffin embedded and sectioned at 5 μm thickness. Every fifth section was collected throughout the entire kidney and non-overlapping images were taken at ×10 magnification. The number of glomeruli was counted from each image and an average was calculated from 4 different mice. Note, P1 kidneys have been previously shown to have more than 10,000 glomeruli; however, the sampling method was significantly different from ours.

**Quantitative PCR.** Cultured kidneys treated with various compounds were cultured for 48 h and stored in RNA-later. A minimum of 9 kidneys for each treatment and genotype were pooled together to isolate RNA using the Qiagen mini kit. cDNA was made using 1.5-2 μg of RNA for all samples using iScript reagents from BioRad. Real-time analysis was performed and amplification was calculated comparing the Ct values of target genes to cyclophilin (used as an internal control) and the fold change by comparing the CT values of untreated versus treated samples. The experiment was carried out three times and the error bar represents the s.e.m.

For rat metanephric mesenchyme qRT-PCR, the following primers were used: Rattus_Six2F: 5'-CAAGAATGAAAGGTGCTCA-3'; Rattus_Six2R: 5'-CTCCTCTCCGCTATGATG-3'; Rattus_Cited1F: 5'-ATGCCAACGAGAGATGAA-3'; Rattus_Cited1R: 5'-TGCCAGTAGAGAGGCGCTGTT-3'; Rattus_Pax8F: 5'-AAGTGCAGATCGGCAGCA-3'; Rattus_Pax8R: 5'-CCAAGGCCAAATGCGTTGC-3'; Rattus_SalIF: 5'-ATCAGGCGGTTGGAAGCGCT-3'; Rattus_SalIR: 5'-TGCCATCTGCTCAGT-3'; Rattus_YAPF: 5'-TGTCACACATCTCAGACTG-3'; Rattus_YARP: 5'-TGTCACACATCTCAGACTG-3'; Rattus_Wnt4F: 5'-AGTGGACAGAAGCATGCAGGATGAG-3'; Rattus_Wnt4R: 5'-TTCGCAAGAGATGAGGCTGATGTTCCAGC-3'; Rattus_E-cadherinF: 5'-TGGTGTCTGATCATTGTGATTTAAG-3'; Rattus_E-cadherinR: 5'-TTGGTGTCTGATCATTGTGATTTAAG-3'; Rattus_Wnt4F: 5'-ATAGGGAGATGTTGCGAGCATG-3'; Rattus_Wnt4R: 5'-ATAGGGAGATGTTGCGAGCATG-3'; Rattus_Tafa5F: 5'-GGAAGTTGGAGGACCAGAACAA-3'; Rattus_Tafa5R: 5'-GGAGTGGGAAGGACCAGAACAA-3'; Rattus_C1qdc2F: 5'-CTCGCTAGTCTGCACTG-3'; Rattus_C1qdc2R: 5'-CTGACGACAGGTCAGTGACTG-3'; Rattus_Cited1R: 5'-CGCCTCGATGTAG-3'; Rattus_Six2F: 5'-CTGACGACAGGTCAGTGACTG-3'; Rattus_Six2R: 5'-CTGACGACAGGTCAGTGACTG-3'; Rattus_Pax8R: 5'-CCAAGGCCAAATGCGTTGC-3'; Rattus_Pax8F: 5'-CCAAGGCCAAATGCGTTGC-3'; Rattus_Cited1R: 5'-CGCCTCGATGTAGT-3'; Rattus_Six2F: 5'-CTGACGACAGGTCAGTGACTG-3'; Rattus_Six2R: 5'-CTGACGACAGGTCAGTGACTG-3';

**Statistical analysis.** Statistical analysis was performed and P values were determined for all quantitative studies using Student’s t-test with equal variance.

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Stromal–epithelial crosstalk regulates kidney progenitor cell differentiation

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Figure S1 Characterization of the stromaless mutants. Panel A: Ablation of the stroma in the Foxd1cre; Rosa26DTA mutant kidneys. Comparison of stromal markers in wildtype (A, C, E) and Foxd1Cre;Rosa26DTA (B, D, F) kidneys at e15.5 (A-D) and 18.5 (E, F). At E15.5, the stromal expression of Foxd1 (green in A and B) cortical to the progenitor cells is lost (arrows in A and B.). Meis1/2 (green in C-F) is decreased in mutants at E15.5 (arrow in D) and completely lost by E18.5 (arrow in F). All sections are co-stained with the progenitor marker Six2 (red) and the UB marker cytokeratin (blue in A-D) or the lectin Dolichous biflorus agglutinin (DBA. White in E, F). In A and B, the cortex of the kidney is up and the medulla is down. In C-F, cortex is to the left, the medulla is to the right. Scale bar = 50 microns. Panel B: Ablation of the stroma affects the activation of distinct classes of Wnt9b target genes in the nephron progenitors. In situ hybridization on sections of E15.5 wildtype (A, C, E, G) and FoxdCre;Rosa26DTA (B, D, F, H) kidneys hybridized with antisense probes to Class II/progenitor targets Tafa5 (A, B) and Pla2g7 (C, D) and Class I/PTA targets Pax8 (E, F) and C1qdc2 (G, H). Note: Expanded domain of Class II targets and significant reduction in Class I. All images captured at 20x magnification. Scale bar = 100 microns.
Figure S2 Localization of YAP in vivo and in vitro. Left panel: Low mag localization of total and phosphorylated Yap: E18.5 wild type (A, B) and Fat4 null (C) kidneys were subjected to immunostaining using YAP and pYAP antibodies (in green). All slides were co-stained with Six2 (red) and Cytokeratin (blue). Single channel images for pYap or Yap are shown in black and white below the corresponding color panel. In all panels, the cortex is up and medullary region is down. Arrowheads in A’ and B’ indicates cortical zone while the arrows indicate the medullary region. Scale bar = 100 microns. Right panel: Expression of YAP in cell cultures: Immunostaining of MM progenitor cells with anti-Yap antibody (green). YAP predominantly exhibits nuclear expression at low density and diffuse expression throughout the cell at higher density. Nuclei are co-stained with Dapi (blue). Scale bar: 20 microns.
**Figure S3** Kinetics of TAZ/YAP ablation from the progenitors. Wildtype (A, C, E, G) and Six2Cre;Yap^flx/flx;Taz^flx/flx (B, D, F, H) kidneys from E11.5 (A, B), 12.5 (C, D), 13.5 (E, F) and 18.5 (G, H) embryos stained with antibodies to pYAP (green), Six2 (red) and Cytokeratin (CK, blue). Single channel images for pYap are shown in black and white to the right of the corresponding color panel. Note efficient ablation of pYap from the Six2 expressing domain by E13.5. Scale bars = 100 microns.
Figure S4 Response of cultured progenitor cells to Wnt and Yap signaling. Mesenchymal cells isolated from 20 embryos from 3 distinct mothers were cultured and subjected to qRTPCR to analyze Wnt9b target gene expression and Six2 after the following treatments: (A) transfection with an empty plasmid or a Lef1/beta-catenin fusion construct; (B) addition of IWP2 or IWR1 to the media for 48hrs. Shown is one representative experiment analysed in triplicate PCR reactions.
Figure S5 Percent proliferation of Six2 positive progenitor cells in wild type, Fat4 and Six2Cre;Tazflox/flox;Yapflox/flox mutants. Kidneys were co-stained with Six2, cytokeratin and pHH3. The number of Six2/pHH3 double positive cells was quantified for wildtype TAZ/YAP and Fat4 mutants. 10.5% (p=0.004) of Six2 positive cells were PHH3 positive in the Six2cre; Tazflox/flox; YAPflox/flox mutants, 16% (p=0.02) in Fat4 mutants and 13% in wild type. Data is the mean from 5 different kidneys for TAZ/YAP mutants (compared to 5 wildtype littermates) and 4 kidneys from Fat4 mutants (compared to 4 wildtype littermates). Statistics source data can be found in Table1. Error bars indicate SEM.
Figure S6 Expression of Class I targets requires a Wnt ligand in wildtype, stromaless and Fat4 mutants. A-F: In situ hybridization analyzing the expression of a Class I/PTA target C1qdc2 in kidney explants isolated from E11.5 wild type (A-B), Foxd1cre; Rosa26DTA (C-D) and Fat4 null (E-F) kidneys treated either with DMSO (A, C, E) or a Wnt ligand production inhibitor IWP2 (B, D, F).
Figure S7 Expression of stromal and progenitor targets in various mutants.
Panel A: The stroma develops normally in Fat4 mutants. E13.5 (A, B, E, F, I, J, M, N) and 18.5 (C, D, G, H, K, L, O, P) wild type (A, C, E, G, I, K, M, O) and Fat4 null (B, D, F, H, J, L, N, P) kidney sections stained with the progenitor marker Six2 (red), the UB/collecting duct marker CK (blue) and the cortical stroma markers Foxd1 (green in A-D) and Meis 1/2 (green in E-H) and the medullary stroma markers Slug (green in I-L) and Lef1 (green in M-P). Panel B: Characterization of Class II/progenitor targets in Wnt9b/Vangl2 double mutants: E15.5 (A-D) and 18.5 (E-H) kidney sections from wild type (A,E), Wnt9b null (B), Wnt9b hypomorph (neo) (F), Vangl2⁻/⁻ (C, G) and Wnt9b⁻/⁻;Vangl2⁻/⁻ mutants were subjected to immunostaining for progenitor genes Cited1 (green), Six2 (red) and the UB marker cytokeratin (blue). Panel C: pSmad1/5/8 expression remains unaltered in wild type and mutant kidneys: E18.5 wildtype (A,B), Six2Cre;Yapflox/flox;Tazflox/flox (C,D) and Fat4⁻/⁻ (E, F) kidneys stained with antibodies to pSmad1/5/8 (green) and the collecting duct marker DBA (red). Images show the cortical regions (A, C, E) and the medullary region (B, D, F). There is no change in pSmad activity in Fat4 or Yap/Taz mutants relative to wildtype. Scale bar = 100 microns.
Figure S8 Characterization of wildtype, Wnt9b, Fat4 and YAP/TAZ mutant kidneys. A: Fate mapping of the nephron progenitors in Wnt9b null kidneys. The Six2 expressing progenitor cells were lineage traced in SixCre;RosaYap (a,c,e,g) and SixCre;RosaYap;Wnt9b−/− (b,d,f,h) kidneys by co-staining with antibodies to GFP (green) and the stromal proteins (in red) Foxd1 (a,b), Meis1/2 (c,d), Slug (e,f) and Lef1 (g,h). The ureteric bud epithelia was stained with anti-cytokeratin (blue) B: In situ hybridization using antisense riboprobes for Taz and Yap on Wildtype and Fat4 null kidneys at E18.5. C: Wildtype (A, E, I), Taz single (B, F, J), YAP single (C, G, K) and Taz and YAP double mutants (D, H, L) were analyzed for the expression of the following targets. Class II/progenitor target Amphiphysin (in red), pYAP (in green) (A-D); Class II target Cited1 (in red) and GFP (in green, indicating Six2cre activity) (E-H); Class I/PTA target Pax8 (in purple) (I-L). The ureteric bud epithelia was stained with DBA (blue in A-H). scale bar 100 microns.
Figure S9  Un-cropped images showing molecular weight markers for all Western Blots included in the main text.
Supplementary Table Legends:

Table S1 List of miR-205 target mRNAs and predicted target sites.