Yap- and Cdc42-Dependent Nephrogenesis and Morphogenesis during Mouse Kidney Development

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Abstract

Yap is a transcriptional co-activator that regulates cell proliferation and apoptosis downstream of the Hippo kinase pathway. We investigated Yap function during mouse kidney development using a conditional knockout strategy that specifically inactivated Yap within the nephrogenic lineage. We found that Yap is essential for nephron induction and morphogenesis, surprisingly, in a manner independent of regulation of cell proliferation and apoptosis. We used microarray analysis to identify a suite of novel Yap-dependent genes that function during nephron formation and have been implicated in morphogenesis. Previous in vitro studies have indicated that Yap can respond to mechanical stresses in cultured cells downstream of the small GTPases RhoA. We find that tissue-specific inactivation of the Rho GTPase Cdc42 causes a severe defect in nephrogenesis that strikingly phenocopies loss of Yap. Ablation of Cdc42 decreases nuclear localization of Yap, leading to a reduction of Yap-dependent gene expression. We propose that Yap responds to Cdc42-dependent signals in nephron progenitor cells to activate a genetic program required to shape the functioning nephron.

Introduction

Nephrons are the functional units of the kidney. Variability in nephron number (300,000 to 1 million in each kidney [1]) in human depends on both environmental and genetic factors. Low nephron number at birth correlates with increased incidence of renal failure later in life [2]. Thus, it is critical to understand the molecular mechanisms underlying nephron induction and patterning.

Kidney organogenesis is a remarkably orchestrated, reiterated process that depends on reciprocal signaling between the epithelial ureteric bud (UB) and the surrounding metanephric mesenchyme [3–6]. Signaling from the mesenchyme induces successive rounds of UB branching, generating the collecting duct system of the kidney. Surrounding the UB are self-renewing mesenchymal progenitor cells called the cap mesenchyme (CM). A subset of CM cells is reciprocally induced by the UB to form a pretubular aggregate (PA), which subsequently undergoes a mesenchyme-to-epithelial transition (MET) to form a renal vesicle (RV). The RV then undergoes morphogenesis, first changing into a comma-shaped body (CSB) that then elongates and folds back on itself to form a S-shaped body (SSB) (Figure 1A). Finally, the SSB further elongates and undergoes patterned differentiation to give rise to the various segments of the nephron (Figure 1A’). This process is repeated thousands of times, resulting in the stereotypical structure of the mature nephron which includes the distal tubules, proximal tubules, Henle’s loops and glomeruli. How this intricate morphogenetic process is regulated is not fully understood.

The Hippo pathway is a highly conserved kinase cassette that regulates tissue growth in metazoans by controlling the activity of Yap and Taz (reviewed in [7–9]). Yap and Taz are closely related transcriptional co-activators that control expression of genes that promote cell proliferation and inhibit apoptosis. When the Hippo kinases Mst and Lats are active, Yap and Taz are phosphorylated and excluded from the nucleus. Loss of Hippo signaling leads to unrestricted proliferation in flies and mammals, and has been linked to a variety of cancers (reviewed in [10], [11]). Yap knockout (Yap⁺/-) embryos die at embryonic day 8.5 (E8.5) [12], and Taz⁻/- mice have polycystic kidney disease [13], [14]. Yap⁻/-;Taz⁻/- embryos die prior to the morula stage with defects in trophectoderm specification, indicating redundant roles in early embryonic development [15].

Blocking the inhibitory effects of Mst/Lats signaling on Yap, either through disruption of Salvador (Sav1/WW45) or by forced expression of a constitutively active form of Yap, leads to hyperproliferation of cells in the gut and skin [16], [17]. Hippo signaling has also been shown to restrict heart size in mice [18]. Upstream of Hippo kinases lie a number of cell surface regulators, which include cadherins, cell polarity complexes and GPCRs [19]. These and other data in flies, fish and mice (reviewed in [7], [8], [20]) have led to a model in which Yap and Taz primarily function to regulate tissue growth.

Surprisingly, recent studies in tissue culture demonstrate that Yap and Taz also respond to mechanical stresses [21], [22]. Plating cells on a rigid substrate induces the nuclear localization of...
Yap and Cdc42 in Nephron Formation

Author Summary

The mammalian kidney undergoes reiterative and stereotypical morphogenetic changes to create the elaborately convoluted adult nephron, the functional filtration unit of the kidney. How these sequential morphological events are controlled remains poorly understood. Here we show that the transcriptional activator Yap is essential in the developing murine kidney. Yap mutants have reduced nephrogenesis and defective morphogenesis. Yap function in nephrogenesis is independent of its previously described role in regulation of cell proliferation and apoptosis. Instead, Yap activity is needed for proper expression of a suite of genes that control cell signaling and cell structure. Remarkably, we find that ablation of Cdc42 phenocopies loss of Yap. We show that Cdc42 is essential for nuclear access of Yap, both in vivo and in tissue culture studies. Taken together, our work shows that Yap and Cdc42 are essential for the cell fate and morphogenesis decisions necessary to shape functioning nephrons, and suggests that Yap functions downstream of Cdc42 during kidney development.

Results

Deletion of Yap in the CM results in abnormal nephron formation

To investigate a potential function of Yap in nephrogenesis, we first stained developing kidneys with antibodies to Yap, and found that Yap was dynamically expressed throughout nephrogenesis. As a transcriptional co-activator the function of Yap is primarily regulated at the level of access to the nucleus [23]. Yap is expressed in the ureteric compartment and cortical stromal cells, with lower levels of expression in the CM (Figure 1B, 1E). Strikingly, we noted that the distribution of Yap is regulated spatially and temporally during nephrogenesis. In early nephrogenic structures, Yap is strongly expressed in proximal cells of the RV (Figure 1B, 1E) and in most distal and proximal cells of the SSB (Figure 1E, 1F–1G). This dynamic expression pattern was seen using two different Yap antibodies, and was lost upon deletion of Yap from the CM using Six2:Cre (Figure 1D and Figure S1).

Yap localization in the nucleus is often regulated by phosphorylation. We stained embryonic kidneys with antibodies that recognize Yap phosphorylated at S127, a site that is phosphorylated by Lats in response to Hippo activation [24], and found that phospho-Yap staining is detectable throughout kidney development (Figure 1C and Figure S2). However, we found no correlation between phospho-Yap staining and Yap localization in the RV or SSB stages.

To directly assess the function of Yap during nephron formation, we removed Yap from the CM with Six2:CreTGc4/4 [25]. Since all components of the nephron, from the glomerulus to the distal tubule derive from Six2-expressing CM cells, this system removes Yap from the CM and all of its epithelial derivatives (i.e. podocyte, Bowman’s capsule, proximal tubule, Henle’s Loop and distal tubule). We found that Six2:CreTGc4/Yapflox/flox (termed YapCM−/−) newborns were obtained at Mendelian ratios. However, despite successful feeding, YapCM−/− animals died within 48 hours of birth. Gross anatomical examination revealed that neonatal (P0) YapCM−/− animals had hypoplastic kidneys and an empty bladder suggesting a failure to produce urine (Figure 1G, 1H). Histological examination of E18.5 kidney sections revealed a smaller papilla and a reduced nephrogenic zone. Convoluted renal tubules and glomeruli were not distinguishable in the inner cortex of the mutant, and the medulla was mainly composed of collecting ducts, suggesting a dramatic reduction in Henle’s loop formation (Figure 11–1L). YapCM−/− mutant kidneys had few detectable glomeruli and proximal tubules (Figure 1K–1N). Strikingly, the rare glomeruli observed in YapCM−/− mutants were ultrastructurally abnormal characterized by simplified capillary tufts ensheathed with podocytes having effaced foot processes (Figure S3).

Labeling with Dolichos Biflorus Agglutinin (DBA) lectin or Calbindin (Figure S4 and data not shown) confirmed that a branched collecting duct system was present as expected, as this structure does not derive from the CM. To determine which nephron compartment was affected by Yap inactivation, we used markers of distinct CM-derived nephron segments. Podocin staining labeled numerous glomeruli in wild-type kidneys, however, considerably fewer podocin-positive structures were detected in YapCM−/− at birth, consistent with the reduced number of glomeruli seen in histological analysis (glomeruli number per section at P0: control:50±5; YapCM−/−:6±2; ***p<0.001. Figure 2A, 2B). Furthermore, the few glomeruli observed in YapCM−/− mice had abnormal structures as seen by triple staining with podocin, WT1 and tomato-lectin (Figure 2C–2D’ and Figure S3). Yap and phospho-Yap antibodies failed to stain the Six2-positive compartments in YapCM−/− (Figure 1D, low and high magnification image panels are shown in Figure S1 and S2), suggesting that Yap excision was efficient, and that the rare nephron derivatives that form in mutants are likely not due to incomplete inactivation of Yap. Examination of markers at E18.5 revealed a dramatic loss of Lotus tetragonolobus lectin (LTL)-positive proximal tubule structures (Figure 2E, 2F). Interestingly, the morphogenesis of the remaining LTL-positive tubules was severely affected as they have barely discernable lumens at E18.5 (Figure 1M, 1N and Figure 2E, 2F). Staining for Ezrin, LTL

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and Par3 was normal in the residual tubules, indicating that cell polarity was retained (Figure S5). The reduced lumens may reflect an absence of filtration due to the dramatically reduced number of glomeruli. Strikingly, YapCM2/2 kidneys also have defects in Henle’s loop (Slc12a1) and distal tubule (Slc12a3) formation (Figure 2G–2J). Thus Yap is necessary in CM cells for normal nephron development.

The CM plays an essential role in supporting branching morphogenesis of the developing kidney. To determine if loss of Yap from the CM alters branching, we analyzed the number of

**Figure 1. Yap is required for kidney development.** (A) Stages of nephrogenesis and their relationship to the UB (black) tips. Signals released from UB tips induce mesenchyme cells to condense around UB tips forming the CM (blue). Some of these CM cells aggregate forming the PA that converts into epithelial RV. The late RV fuses with UB tips and develops into comma (CSB) and S-shaped (SSB) body. (A’) Schematic diagram of the nephron components. (B) Confocal images for Yap, E-cadherin and DAPI staining in late RV at E14.5. Nuclear Yap is observed in the proximal segment of the RV (arrowheads), while expression disappears in Six2:Cre expressing cells (D - arrows point to CM cells, arrowhead points to an early nephron). (C) Confocal images of p-Yap/E-cadherin/DAPI staining shows ubiquitous p-Yap expression. Individual channels images are in Figure S2. (E) Immunohistochemistry using Yap/Taz antibody in RV and SSB shows a similar expression pattern observed with Yap antibody in previous panels (arrowheads). (F–F’) Confocal images for Yap/E-cadherin/DAPI staining in SSB at E14.5. Nuclear Yap is observed in proximal and distal segments of the SSB (arrowheads). (G,H) Macroscopic view of the urogenital system from wild-type and Yap mutant kidneys at P0. Note bilateral reduction in kidney size of mutant compared to control and empty bladder in mutant animals. (I,J) PAS staining of P0 kidneys from wild-type and YapCM2/2 animals. Arrows point to the papilla. (KL) Closer view of the cortical zone shows limited nephrogenesis in YapCM2/2. (MN) Higher magnification shows abnormal glomeruli structure and tubules with barely discernable lumens (asterisk) in YapCM2/2. k: kidney; b: bladder; cd: collecting duct; csb: comma-shaped body; d: distal; g: glomeruli; ic: inner cortex; ma: medulla; m: medial; nz: nephrogenic zone; p: proximal; pt: proximal tubule; ssb: S-shaped body. Scale bars represent 25 μm (B–F’; M–N), 1 mm (G–J), 200 μm (K,L). doi:10.1371/journal.pgen.1003380.g001

**Figure 2. Loss of CM-derived epithelial structures and abnormal morphogenesis in Yap mutants.** (A–J) Sections of P0 kidneys stained using late nephron markers confirm abnormal nephron formation in YapCM2/2 kidneys. Glomeruli (Podocin, A,B; Podocin-WT1-Tomato lectin, C–D’). Proximal tubules (LTL, E,F). Henle’s loop (Slc12a1, G,H). Distal tubules (Slc12a2, I, J). (K,L) Overview of an E14.5 nephrogenic zone reveals the presence of CM cells (arrows) in both genotypes, but CM-derived epithelial structures (arrowheads) are greatly reduced in mutant when compared to control littermates. (M–N’) Higher magnification shows histological morphology defects of mutant SSB compared to wild-type controls at E13.5. Scale bars represent 500 μm (A,B), 50 μm (C–D’), 200 μm (E–J), 100 μm (K–L). doi:10.1371/journal.pgen.1003380.g002
ureteric tips at different time-points using immunofluorescent staining with antibodies to Calbindin, which marks both the CD and the UB tips (Figure S4). While similar branching is observed in wild-type and Yap\textsuperscript{CM/-} kidneys at E14.5, the number of UB tips slightly decreases at E16.5 in Yap mutants with a significant reduction of tip number at P0. Thus loss of Yap in the CM does not affect early branching but has late-onset impairment of branching.

**Yap deletion impairs nephrogenesis and S-shaped bodies’ morphogenesis**

To determine the developmental origin of the defective nephrogenesis in Yap\textsuperscript{CM/-} kidneys, we examined kidney development from E13.5 to E18.5. Nephrogenesis occurs in a repetitive manner, with new nephrons being formed throughout development at the outer cortex of the kidney. This process is highly regulated, involving both inductive and repressive signals (reviewed in [4]). Six2-expressing precursor cells residing in the cortex self-renew to replenish a pool of mesenchymal cells that are then transformed into nascent nephrons [25]. Maintenance of the progenitor population requires Six2, as deletion of Six2 results in premature differentiation of the CM cells [23]. Histological analysis revealed that Yap\textsuperscript{CM/-} kidneys have limited nephrogenesis (Figure 2K, 2L) with abnormal morphogenesis of SSB (Figure 2M–2N). CM cells in both Yap\textsuperscript{CM/-} and wild-type kidneys were detected by histological analysis at E14.5 (Figure 2K–2N) and by Six2, Gdnf and Sall1 expression (Figure 3A, 3B, 3F–3K), indicating that nephrogenic precursors cells are present in Yap mutant kidneys. Clear Six2 staining is obvious even at P0 (Figure 9W), although there is a mild reduction in the total number of Six2-positive cells in Yap mutant kidneys compared to wild-type kidneys (Figure S6). In contrast, however, the number of nascent nephrons (PA, RV, CSB and SSB) was clearly and dramatically reduced in Yap mutant kidneys early in development, as revealed by histological analysis (Figure 2K, 2L), NCAM staining (Figure 3C, 3D) and WT1 staining (Figure 3H, 3I). Quantification of NCAM-positive nephrogenic structures at E15.5 further validated a significant decrease in total nephrogenesis due to Yap deletion (Figure 3E). Since no change in branching morphogenesis could be detected at this stage (Figure S4), the limited nephrogenesis in Yap mutants is not secondary to impaired ureteric branching. Few PA could be detected in Yap mutant kidneys (Figure 3E), further showing that nephron induction is impaired.

**Figure 3. Yap deletion impairs nephron induction, without affecting self-renewal of the CM population.** (A,B) Immunostaining analysis for Six2 (E14.5) shows no change in expression pattern in both genotypes (arrows). E-cadherin was used to visualize the UB compartment. (C,D) Dramatic reduction in nephrogenesis visualized by loss of NCAM-expressing structures (arrowheads) in the nephrogenic zone of Yap mutant compared to wild-type (E16.5). Note the reduced NCAM expression in CM cells. Calbindin highlights the UB and CD. (E) Quantification of early nephron structures in E15.5 controls (black columns) and Yap mutants (white columns) based on NCAM staining. Total***: p < 0.0001; PA***: p < 0.0001; RV*: p = 0.0209; CSB*: p = 0.0018; SSB***: p < 0.0001. (F,G)ISH analysis shows maintained Gdnf expression in CM of control and Yap mutants (E15.5). (H,J) WT1 staining (E18.5) reveals staining in CM cells (arrows) for both genotypes, and dramatic reduction in number of renal MET-derived structures in mutants compared to wild-type. (I,K) Immunostaining analysis for the CM marker Sall1 (E14.5) shows no change in expression pattern in both genotypes. E-cadherin was used to visualize the UB compartment. Scale bars represent 100 μm.

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severely disrupted. In addition, the number of CM derived structures that reached the SSB stage in the mutant was dramatically reduced when compared to controls (Figure 3E). Thus, while the self-renewing capacity of CM cells is largely Yap independent, Yap-depleted CM cells are less potent to undergo nephrogenesis, and are unable to execute regulated morphogenesis to form regular SSB.

**Segmentation of the renal vesicle is independent of Yap function**

Formation of a functioning nephron requires polarization of the emerging epithelium along a proximodistal axis to specify diverse cell types. Polarization and segmentation is detectable as early as the RV stage. Segmentation becomes clearly apparent at the SSB stage with distal-, medial- and proximal-specific gene expression. We examined nephron segmentation at both the RV and SSB stages. Yap deletion did not impair RV polarization as proximal (WT1) and distal (E-cadherin, Hnf1ß, Sox9, Jag1) markers showed similar expression patterns (Figure 4A–4H). Similarly, in later nephrogenic structures, no segmentation defect could be seen in distal and medial SSB (Figure 4A–4H, Distal:E-cadherin, Hnf1ß, Sox9, Medial:Jag1, Hnf1ß, Sox9, Proximal:WT1) [26–28]. However, Yap-null SSB have a reduced WT1 positive proximal segment (in particular compare Figure 4G versus 4D; 4E versus 4F) consistent with defects in proximal fates seen in P0 kidneys.

Formation of a functional nephron also requires fusion to the ureteric bud, a process that occurs at the late RV stage [29]. Staining with Laminin to mark the basement membrane (BM) and Cytokeratin to mark the ureteric epithelium (UE) of an early RV shows that the RV is surrounded by its own BM and separated from the adjacent UE by the ureteric epithelial BM in both controls and Yap(Gm/-) mutants (Figure 4I, 4J). At the comma stage (Figure 4I, 4J) fusion of the early nephron to the UE is complete in both genotypes as seen by a continuous BM. However, we note that Yap(Gm/-) mutants consistently display aberrant morphology at the connecting segment, where the SSB connects to the UE (asterisk, see also Figure 3K, Figure 5F, Figure S2B). In particular, the distal segment of the SSB does not correctly merge with the outermost edge of the UB.

**Loss of Yap does not significantly impact cell proliferation or apoptosis during early nephrogenesis**

Yap, downstream of the Hippo pathway, has been extensively shown to regulate organ size by promoting cell proliferation and inhibiting apoptosis. We therefore analyzed cell proliferation throughout nephrogenesis to ascertain if altered proliferation or apoptosis could explain the morphological defects in Yap mutants. Quantification of BrdU incorporation in nephron progenitors cells (Six2 positive cells) did not reveal any significant changes in CM proliferation (n = 1,000 Six2 positive cells from 4 kidneys of each genotype, Figure 5A, 5B and 5G). Interestingly, while no significant changes could be detected in overall RV proliferation, or in distal RV proliferation, a slight reduction in proliferation was detectable in the proximal part of Yap-null RV (Hnf1ß negative, n = 16 RV per genotype - Figure 5C, 5D and 5G). Finally, we investigated proliferation in the distal (cells located between the UE and Jag1 expressing domain), medial (Jag1 positive cells) and proximal segments of the SSB (n = 12 SSB per genotype). Similarly to the RV stage, no significant change in proliferation could be detected in the overall SSB, however segment-specific analysis revealed slightly decreased proliferation in the distal segment of Yap mutant (Figure 5E, 5F and 5G). TUNEL staining in control and Yap(Gm/-) kidneys (E18.5) did not reveal any changes in apoptosis in mutants relative to controls (Figure 5H, 5I). Our data indicates that early defects in nephron formation in Yap mutants are not due to death of the nephrogenic cell population, nor to a dramatic failure to proliferate.

**No major disruptions of Notch or Wnt/ß-catenin signaling in Yap(Gm/-) kidneys**

Recent studies have revealed functional interactions between Yap and β-catenin, [18], [30]. While activation of the canonical β-catenin signaling pathway is necessary for nephron formation, its repression is required for epithelialization to occur [31]. Wnt5b secreted from the UB induces mesenchymal condensation via canonical β-catenin signaling, activating a molecular cascade involving Fgf8, Wnt4, Pax8 and Lmm1 [32]. Expression of Wnt5b was unchanged in Yap mutant kidneys (Figure S7A, S7B). To see if β-catenin signaling was affected in Yap mutant kidneys, we examined expression of β-catenin signaling targets. Significantly, expression of the established Wnt target genes Pla2g7, Ctgf, 2 and Lef1 were unchanged in Yap(Gm/-) kidneys (Figure S7C–S7H). Moreover, Fgf8, Wnt4, Pax8, and Lmm1 expression levels were also unchanged in Yap(Gm/-) kidneys (Figure S7I–S7P). Finally, removing one allele of β-catenin in Yap(Gm/-) mice (by generating Six2(Cre)Yaplox[lox]/Yaplox[lox]-/-cattenin(KO)lox[lox]/+ embryos) does not alter the Yap(Gm/-) phenotype (Figure S8). Taken together, these data indicate that Yap functions in nephron formation independently of changes in the WT/β-catenin signaling pathway.

The defects in glomeruli and proximal tubules that occur in Yap(Gm/-) kidneys are reminiscent of defects in Notch signaling [27]. We therefore assayed different members of the Notch pathway by in situ hybridization (ISH). In particular, no changes were detected in the expression levels of Notch1, Notch2, the ligand Jagged1, or the Notch targets Hes1 or Hes5 (Figure S7Q–S7X and data not shown). These data indicate that the loss of Yap does not lead to nephrogenic defects via loss of Notch signaling.

**Whole-genome expression analysis identifies novel Yap-dependent genes in the kidney**

Since no defects were observed in β-catenin or Notch signaling, and proliferation and apoptosis were largely unaffected, we sought an unbiased approach to determine the molecular basis of the defects seen in Yap mutants. We used whole-genome transcript profiling (Mouse Whole Genome-6 v2.0 BeadChip) at E13.75 to determine gene expression changes in Yap mutant kidneys. Of the ~45,000 transcripts represented on the array, 334 genes were found to be differentially expressed in Yap(Gm/-) kidneys (fold change>1.27, p-value<0.05). We used both Genepaint (www.genepaint.org) and Gudmap (www.gudmap.org) databases to examine candidate expression in the developing kidney. This approach allowed us to concentrate on 24 candidates (Table S1). To confirm changes in Yap(Gm/-) mutants, we performed ISH and antibody staining in E14.5 control and Yap(Gm/-) kidneys. Our analysis confirmed that expression of Cited1, Meox2, Traf1 and Capn6 was lost in Yap-null CM cells (Figure 6D–6O). Similarly, expression of Pax2, Uncx4.1 and Switch1 were significantly reduced in Yap mutants (Figure 6A–6C, and Figure S9A–S9F). While Fgfg1 expression was barely detectable in wild-type CM cells, strong mesenchymal expression of Fgf10 was observed in Yap(Gm/-) kidneys (Figure 6P–6R). Surprisingly, expression of both Ret and Retl was greatly increased respectively in UB tips and collecting ducts of Yap knockout kidneys, indicating that loss of Yap in the CM autonomously affects expression of these genes (Figure S9G–S9L). This work identifies a set of genes that depend on Yap expression during nephron development that function in differentiation and morphogenesis rather than proliferation and apoptosis.
Figure 4. Characterization of segmentation in Yap mutant nephrons. (A–B') Double staining for E-cadherin and Calbindin in RV and SSB. Co-staining for Hnf1ß/WT1 (C–D') and Sox9/WT1 (E–F') reveals normal segmentation of the RV with both proximal and distal segments. Similarly, SSB show normal segmentation. Note the reduced size of the proximal domain in Yap-null SSB (compare WT1 positive segment in Yap mutants (D', F') to controls (C', E'). This is also apparent in B' and J'. (G–H') Immunofluorescence for E-cadherin and Jag1 reveals no change in specification of the distal RV and the medial segment of the SSB in both genotypes. Note the aberrant morphology (asterisk) of the site where the connection occurred between the SSB and the UE (B', D', F', H' and J'). (I–J') Immunofluorescence using antibodies to Cytokeratin (UE) and Laminin (BM) shows that fusion occurred before the comma-shaped stages. All staining performed at E15.5. CSB: comma-shaped body; RV: renal vesicle; SSB: S-shaped body. Scale bars represent 25 μm. DAPI was used to counterstain nuclei.
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Inactivation of Cdc42 phenocopies loss of Yap

Staining with antibodies to phospho-Yap did not indicate any obvious spatial or temporal regulation by Hippo kinases that could explain the regulation of Yap localization or activity during nephrogenesis (Figure 1B, 1E, 1F and Figure S1). We therefore searched for other potential regulators of Yap activity. Recent studies in cultured mammalian cells have demonstrated that Yap can be regulated in a Hippo kinase independent manner by mechanical signals exerted by extracellular matrix rigidity and cell shape [21]. Mechanical signals regulate Yap localization via small GTPase activity and the actin cytoskeleton. Cdc42 is a conserved and critical regulator of the actin cytoskeleton, acting through Arp2/3 and N-Wasp [33]. To examine the role of Cdc42 in nephrogenesis, we used Six2:Cre to delete Cdc42 from the CM population (Cdc42CM−/−). Loss of Cdc42 from the CM resulted in a severe defect in kidney development that was strikingly similar to YapCM−/−, with hypoplastic kidneys with empty bladders indicating lack of functional nephrons (compare Figure 7A, 7B to Figure 1G, 1H). The histology of E18.5 Cdc42CM−/− kidneys strikingly resembles that of YapCM−/− with a distinctively reduced nephrogenic zone and a smaller papilla (Figure 7C, 7D). Convoluted renal epithelia and glomeruli were absent in the cortex of the mutant (Figure 7E, 7F). Staining with Podocin and quantification of glomeruli demonstrated a significant reduction in glomerular number in Cdc42CM−/− (glomeruli number per section at P0: control:51±2; Cdc42CM−/−:2±2; ***p<0.001), similar to that seen in YapCM−/− kidneys (Figure 7G, 7H). Cdc42CM−/− kidneys also have fewer and truncated proximal tubules with barely discernable lumens (Figure 7I, 7J). Similar to YapCM−/−, nephrogenic precursors are present in Cdc42CM−/− (seen by PAS staining, Six2, San1 and WT1 expression; Figure S10A-S10H), but the capacity of these cells to undergo nephrogenesis is dramatically reduced (NCAM staining - Figure 7K, 7L and Figure S10A, S10B). Together these data show a remarkable similarity between the effects of loss of Yap and the loss of Cdc42 in the CM, suggesting they might function together in kidney development.

Cdc42 is necessary for Yap localization

The primary mechanism of regulating Yap activity is controlling Yap nuclear localization. We therefore tested if loss of Cdc42 affected Yap nuclear localization in developing kidneys. Detailed examination of Cdc42CM−/− kidneys revealed reduced nuclear Yap in Six2 positive CM cells at E12.5 (Figure 8A–8B”). Quantification using ImageJ software further confirmed a significant decrease of nuclear Yap in mutant CM cells compared to wild-type, while no change in the levels of Yap were observed in Cdc42lox/lox embryos. Cdc42lox/lox MEFs were infected with an adenovirus expressing Cre. Yap staining is predominantly in the nuclei of adjacent UB cells (Figure 8E). The small GTPase RhoA has been shown to regulate Yap nuclear localization in mammalian tissue culture [21], [22], however no studies to date have examined the effects of Cdc42 on Yap localization. To better visualize changes in Yap localization upon removal of Cdc42, we examined cultured mouse embryonic fibroblasts (MEFs) isolated from E13.5 Cdc42lox/lox embryos. Cdc42lox/lox MEFs were infected with an adenovirus expressing Cre. Yap staining is predominantly in the nucleus in isolated control MEFs (Figure 8C–8C”), while removal of Cdc42 in MEFs results in more diffuse Yap staining, with reduced nuclear accumulation (Figure 8D–8D”), lower magnification in Figure S11). Thus, loss of Cdc42 in MEFs, as in embryonic kidneys, leads to a decrease in nuclear Yap, indicating that Cdc42 function is necessary for Yap to be normally localized in the nucleus.
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**Control**  
**Yap\(^{CM/-}\)**

**A**  
**B**

**C**  
**Pax2**

**D**  
**E**

**F**  
**Yap**

**G**  
**H**

**I**  
**Meox2**

**J**  
**K**

**L**  
**Traf1**

**M**  
**N**

**O**  
**Capn6**

**P**  
**Q**

**R**  
**Fgf10**
Loss of Cdc42 leads to loss of Yap-dependent gene expression

The remarkable phenotypic similarities of Yap<sup>CM−/−</sup> and Cdc42<sup>CM−/−</sup>, coupled with the observation that loss of Cdc42 leads to reduced levels of nuclear Yap, suggested the hypothesis that Cdc42 is necessary for Yap-dependent gene expression. We tested this hypothesis by examining expression of Yap-dependent genes in Cdc42<sup>CM−/−</sup>, by immunofluorescence and ISH. Staining of E14.5 Cdc42<sup>CM−/−</sup> kidneys revealed dramatic loss of Cited1, Capn6, and Traf1, and a clear reduction of Pax2, Uncx4.1 and Meox2 (Figure 8F–8M and Figure S10I–S10L). In addition, there were marked increases in Fgf10 expression in Cdc42<sup>CM−/−</sup> mutants

Figure 6. Transcriptional changes in Yap mutant CM progenitors cells. Expression of Pax2 (A), Cited1 (D), Meox2 (G), Traf1 (J) and Capn6 (M) in control E14.5 kidneys, demonstrating expression in CM cells and other lineages. Yap deletion results in loss of gene expression of these genes in CM cells (BEHKN). Note the loss of Pax2 expression in the CM of Yap mutant (arrows in B) compared to control CM (arrows in A), while expression in the ureteric epithelium (arrowheads) remain unchanged. (P,Q) ISH reveals increase in levels of Fgf10 expression specifically in nephron progenitor cells of Yap deficient kidneys compared to wild-type. (CFJLOR) Graphical representation of the microarray data of control (black columns) and Yap mutant (white columns). (** p<0.001; *** p<0.0001). Scale bars represent 100 µm.

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Figure 7. Loss of Cdc42 phenocopies Yap<sup>CM−/−</sup> phenotype. (A,B) Macroscopic view of the urogenital system from wild-type and Cdc42<sup>CM−/−</sup> kidneys at P0. Note the reduction in kidney bladder size in mutant animals. (C–F) PAS staining (P0) from wild-type and Cdc42<sup>CM−/−</sup> animals showing smaller papilla (arrows), dramatic reduction of both CM-derived epithelial structures and glomeruli in the mutant. (G–J) Sections of P0 kidneys using late nephron-specific markers confirms the abnormal glomeruli and proximal tubules formation in Cdc42 mutant kidneys. Glomeruli (Podocin, G,H). Proximal tubules (LTL, I,J). (KL) NCAM staining (E15.5) reveals dramatic reduction in the number of CM-derived structures (arrowheads) in mutants compared to wild-type. k: kidney; b: bladder; g: glomeruli; pt: proximal tubule. Scale bars represent 1 mm (A–D), 200 µm (E–L).

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Yap and Cdc42 in Nephron Formation

**A, B, B′, B″, B‴**

Control

**A′, A″, A‴**

Cdc42

**C, C′, C″, C‴**

Cdc42

**C′, C″, C‴**

+ Adeno-Cre

**E**

Fluorescence intensity

**CM nuclei**

Control

Cdc42

**J**

Control

**K**

Cdc42

**F, G, H, I, L, M, N, O**

Cited1 - E-cad

**Caprin6**

Fgf10

**Tal1**

**Mef2c**

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Figure 8. Cdc42 is necessary for Yap to be normally localized and active. (A–B) Staining for Six2 and Yap shows reduce nuclear Yap staining in most of the Six2 positive cells (arrows) of Cdc42flox/flox compared to wild-type at E12.5. Control (C–D) and Cre infected (D–E) Cdc42flox/flox mouse embryonic fibroblasts (MEFs) stained with Yap antibody and doubly counterstained with phalloidin and Hoechst 33258. (E) Quantification from panels A–B of Yap nuclear staining in CM and UB cells from controls (black columns) and Cdc42flox/flox (white columns) kidneys at E12.5. Data represent mean fluorescence intensity per nucleus area (100 nuclei for each genotype - ***p<0.0001). (F–M) Expression of Cited1 (F), Capn6 (H), Traf1 (J), Mox2 (L) in control E14.5 kidneys, demonstrating expression in nephron progenitor cells. Cdc42 deletion results in loss of expression of these genes in CM cells (G, I, K, M), similar to what is seen in YapIRIR mutants. (N,O) ISH reveals increase in levels of Fgf10 expression specifically in CM cells of mutant kidneys compared to wild-type controls. Scale bars represent 25 µm (A–B’), 10 µm (C–D’), 100 µm (F,G), 200 µm (H–O).

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Yap and Taz have distinct functions during nephron development

As described above, YapIRIR mice have dysplastic kidneys with minimal nephrogenesis. The Yap paralogue Taz is required for proper kidney development since TazIRIR mice have cystic kidneys [13,14].

To investigate the function of Taz in the Six2 progenitor cells, we generated Six2:CreTGC/+; Tazflox/flox mice (TazIRIR). Macroscopic analysis of TazIRIR kidneys (Figure 9F, G) showed reduced kidney size compared to controls (Figure 9B), which is similar to what is seen in TazIRIR kidneys (Figure 9H). Histology at P0 reveals highly cystic tubules in the cortex of TazIRIR kidneys (Figure 9I, J), with increased numbers of cystic glomeruli (Figure 9K, L). TazIRIR kidneys were superficially normal (Figure S12). Further studies are needed to fully ascertain the contribution of Mst and Lats kinases to nephrogenesis.

To determine if the residual glomeruli and proximal tubules that form in YapIRIR kidneys are due to a low level redundancy by Taz, we generated Six2:CreTGC/+; Tazflox/flox; Yapflox/flox mice. Significantly, TazIRIR; YapIRIR double mutants showed no exacerbation of glomeruli or proximal tubules deficits relative to YapIRIR mutants (Figure 9I–L) and data not shown). However, some of the few proximal tubules that formed were cystic (Figure 9I, L) similar to Taz single mutants. Together, these data indicate that Yap and Taz play distinct roles during nephrogenesis.

Discussion

Yap and Taz have well-described roles in the regulation of cell proliferation and apoptosis. Here we show that loss of Yap leads to defects in nephron formation and morphogenesis during renal development. We demonstrate that these defects occur independent of major changes in apoptosis or proliferation, and identify a novel set of Yap-dependent genes implicated in morphogenesis.

Yap and Taz are necessary for distinct programs in nephron formation

Yap and Taz are closely related transcriptional co-activators that have been shown in many systems to have similar, and at times partially redundant roles in control of cell proliferation and apoptosis. A striking finding of our in vivo analysis is that loss of Yap leads primarily to misregulation of genes involved in cell fate and morphogenesis. Another surprising finding in our study was the discovery that Yap and Taz play distinct roles during kidney development. While loss of Yap leads to reduced nephrogenesis, with clear morphological defects at the SSB stage, loss of Taz leads to normal sized kidneys, with functioning nephrons, as indicated by a full bladder at birth. Proximal tubules in the Taz mutants are cystic, while the proximal tubules in Yap mutants have barely discernible lumens. Moreover, TazIRIR; YapIRIR double mutants show both loss of glomeruli and proximal tubules, with some tubules becoming dramatically dilated - underscoring the independence of the Taz and Yap phenotypes.

Yap function in early nephron formation is independent of proliferation and apoptosis

Surprisingly, we did not detect any significant changes in proliferation or apoptosis in Yap mutants indicating that in nephrogenesis Yap is functioning independently of previously described roles. We did not detect any spatial regulation of Hippo-dependent Yap phosphorylation. Moreover, we found that both Six1 and Mst1/2 (Pax2;CreIRIR; Mst1IRIR; Mst2flox/flox) knockout kidneys were superficially normal (Figure S12). Further studies are needed to fully ascertain the contribution of Mst and Lats kinases to nephrogen development.

Although Yap is not essential for proliferation or apoptosis in early nephron development, we cannot exclude a later role for Yap in cell proliferation in the tubules. In fact the extremely short tubule segments seen in Yap mutants may reflect a role for Yap in later proliferation. Consistent with this possibility, we found that forced overexpression of Yap in adult kidneys leads to increased proliferation (data not shown). This later role of Yap during nephrogenesis may be controlled by the Hippo pathway, but further examination is required to examine this possibility.

Yap-dependent genes are involved in cell signaling and morphogenesis

Using microarray analysis, we identified a number of novel Yap-dependent genes that function in nephrogenesis. Unexpectedly, these genes were not involved in control of apoptosis and proliferation, but instead involved in cell fate and morphogenesis. A subset of these genes are involved in controlling cell shape and the cytoskeleton. Cored, for example, bundles and stabilizes microtubules [34]. Other genes, such as Sostdc1 and Fgf10 are involved in cell-cell signaling, whereas others such as Mox2 and Cited1 are markers of the early steps of nephrogenesis, and linked to stem cell renewal. Yap has been previously linked in multiple systems with stem cell proliferation and stem cell pluripotency.
Figure 9. Yap and Taz have distinct roles during nephrogenesis. (A–D) Macroscopic view of the urogenital system from wild-type, Taz\(^{CM-/-}\), Yap\(^{CM-/-}\) and Taz\(^{CM-/-}\);Yap\(^{CM-/-}\) double mutant kidneys at P0. (E–H) PAS staining of P0 kidneys. (I–L) Closer view of the cortical zones. (M–P) LTL staining for each genotype. (Q–T) NCAM staining for all genotypes. (U–X) Six2 staining reveals progenitor cell population in all genotypes. Scale bars represent 500 µm (A–H) and 100 µm (I–X).

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Yap nuclear localization is regulated by mechanical signals exerted by extracellular matrix rigidity and cell shape [21]. Notably, recent studies have shown that Cdc42 is essential for matrix contraction in 3D tissue culture assays. Loss of Cdc42 may result in defects in nephrogenesis due to loss of cytoskeletal tensions between the matrix and the tubules as they form, twist and bend during nephrogenesis. We propose that the dramatic changes in cell shape as cells aggregate, epithelialize and contract during formation of nephrons, generates mechanical stresses that are sensed via the cytoskeleton, leading to changes in the nuclear localization of Yap. Once in the nucleus, Yap then promotes expression of genes that are necessary for subsequent steps in nephron formation. While this idea is appealing, clearly much more work is needed to understand how loss of Cdc42 leads to disruption of Yap localization, and changes in Yap-dependent gene expression.

We have shown here that loss of Cdc42 leads to loss of Yap-dependent gene expression and loss of nuclear Yap localization. Taken together, our data suggest a model in which Cdc42 function is necessary for Yap localization and activity during development to shape functioning nephrons.

Materials and Methods

Mouse lines
Cdc42<sup>lox/lox</sup> [38], Mst1<sup>lox</sup> and Mst2<sup>−/−</sup> [39], N-Wasp<sup>lox</sup> [40], Pax2:GRO<sup>Rt</sup> [41], Smo<sup>−/−</sup> [17] and Six2;Grg<sup>Tg/Crt</sup> [25] mouse strains have been described. Yap<sup>lox</sup> and Taz<sup>lox</sup> alleles were generated by inserting LoxP sites for Cre-mediated excision flanking exons 2 as described in Figure S15. All mice were maintained on a mixed genetic background. Husbandry and ethical handling of mice were conducted according to guidelines approved by the Canadian Council on Animal Care. Embryos were genotyped by standard PCR protocol. Genotyping was done by PCR using genomic DNA prepared from mouse ear punches.

Histological and immunological analyses
Embryonic samples from timed matings (day of vaginal plug = E0.5) were collected, fixed in 4% paraformaldehyde overnight at 4°C, serially dehydrated and then embedded in paraffin. Microtome sections of 7 μm thickness were examined histologically via periodic acid-Schiff staining.

For immunofluorescent analysis, paraffin sections were dewaxed and re-hydrated via ethanol series. Antigen retrieval was performed by boiling the sections for 20 minutes in Antigen Unmasking Solution (H-3300, Vector). Sections were incubated for 1 hour in blocking solution (3% BSA, 10% goat serum, 0.1% Tween20 in PBS) at room temperature. Blocking solution was replaced by a solution of primary antibodies diluted in 3% BSA, 3% goat serum, 0.1% Tween20 in PBS. The following primary antibodies were used in this study: Calbindin (PC253C, Calbiochem), Cited1 (RB-9219-P0, Neomarkers), Cytokeratin (F3418, Sigma), E-cadherin (Mouse, 610181, BD Transduction Laboratories), E-cadherin (Rabbit, #3195, Cell Signaling Technology), Ezrin (sc-58758, Santa Cruz Biotechnology), FoxD1 (gift from Andrew P. McMahon (Harvard University, Cambridge), Hnf1β (sc-2280, Santa Cruz Biotechnology), Jag1 (#2620, Cell Signaling Technology), Laminin (L9393, Sigma), LTL (FL-1321, Vector Laboratories), NCAM (C9672, Sigma), Par3 (07-330, Millipore), Pax2 (PRB-276P, Covance), Phospho-Yap (#4911, Cell Signaling Technology), PKG (sc-216, Santa Cruz Biotechnology), Podocin (P0372, Sigma), Six2 (11562-1-AP, Proteintech), Sox9 (AB5535, Chemicon), Tomato-lectin (TL-1176, Vector Laboratories), WT1 (G-19, Santa Cruz Biotechnology), Yap (sc-101199, SantaCruz Biotechnology), Yap/Taz (#8418, Cell Signaling Technology). Relevant Cy3- or FITC-conjugated secondary antibodies (Jackson
were prepared and sectioned as described above before being decapitated. Kidneys were quickly removed and flash frozen in liquid nitrogen and stored at −80°C. E13.75, embryos collected in ice-cold PBS and immediately snap frozen in 0.1 M cacodylate buffer containing 4% paraformaldehyde and 2% glutaraldehyde. Subsequently, P0 kidneys were postfixed in 1% OsO4, dehydrated, and embedded in Quetol-spurr resin. Ultrathin resin sections stained with uranyl acetate and lead citrate were viewed using an FEI CM101 transmission electron microscope (FEI, Hillsboro, OR).

**Glomerulus quantification**

P0 kidneys were dissected in PBS, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Immunostaining against Podocin was performed on P0 median kidney sections and glomeruli were counted. Means were calculated per kidney and genotype. An unpaired two-tailed t-test was used to determine the statistical significance among genotypes.

**Quantification of nuclear Yap**

Quantification of Yap nuclear staining was performed using Image J software. Images were imported into Image J, and, by using DAPI staining to mark the cell nuclei, nuclear Yap signal was measured. The mean signal was calculated from 100 cells for each genotype. An unpaired two-tailed t-test was used to determine the statistical significance.

**BrdU incorporation**

BrdU solution containing 5-Bromo-2′-deoxyuridine (10 mg/ml) was injected intrauterinely in pregnant mice (50 mg BrdU/kg of mice) 2 to 3 hours before embryonic dissection. The samples were prepared and sectioned as described above before being incubated overnight with anti-mouse BrdU antibody (Clone Bu20a, Dako).

**TUNEL**

Terminal deoxynucleotidyl transferase, mediated dioxygenin-deoxyuridine nick end labeling (TUNEL) was performed using the Roche Cell Death Detection Kit on E18.5 kidney sections. The samples were stained with hematoxylin and mounted in pertex.

**In situ hybridization**

Embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C, and then paraffin embedded. Further processing of the embryos and ISH were carried out as described [42]. Riboprobes for Capn6 [34], Raldh3 [43], Fgf10 [44], Ret (gift from Frank Costantini), Slc12a1 and Slc12a3 (gift from A. Brandl), Mox2, Traf1 and Uncx4.1 (from the SLRI Open Freezer) were used [45].

**Supporting Information**

**Figure S1** Efficiency of the Sin2-Cre deletion on Yap conditional allele. Low (A–B′) and high (C–D′) magnification of Yap antibody staining in control (A,C) and Yap mutants (B,D) confirms complete Yap deletion within both CM cells (arrows) and early nephron (arrowheads) in the mutant (D–D′), whereas staining in the UE and stromal compartments persists. (E,F) Specificity of Yap staining is confirmed by lack of staining in negative controls. (E) Sections were stained with mouse immunoglobulin (IgG) and anti-mouse Cy3 secondary antibody, but inclusion of anti-rabbit Cy3 (F) No primary antibody added, with anti-mouse Cy3. (G) Immunohistochemistry using Yap antibody reveals same pattern of expression as seen with IF. (H–J) Immunohistochemistry of Yap/Taz antibody on Yap(CAM−/−), Taz(CAM−/−) and double Yap;Taz mutants demonstrating that staining seen in CM cells and early nephrons is specific to Yap in our system as it disappears in Yap single mutants only (H), but is still present in Taz mutants (I).

**Figure S2** Efficiency of the Sin2-Cre deletion on Yap conditional allele. Low (A,B) and high (C–D) magnification of Yap antibody in control (A,C) and Yap mutant (B,D) confirms complete Yap deletion within CM cells (arrows) in Yap mutant (D–D′), whereas staining in the UE and stromal compartments persists. (E,F) Staining specificity is demonstrated by lack of staining in relevant controls. (E) No Yap primary antibody, but inclusion of anti-rabbit Cy3. (F) Staining with rabbit immunoglobulin and anti-rabbit Cy3.

**Figure S3** Loss of foot processes in Yap mutants. Transmission electron micrographs confirm abnormal glomeruli structure with foot process (arrows) effacement in P0 Yap mutant compared to controls. cc, endothelial cells, p: podocyte. Scale bars represent 10 μm (A,B) and 2 μm (C,D).
Figure S4 Branching morphogenesis in Yap mutants. Staining for Calbindin at E14.5 shows similar number of epithelial tips at E14.5 (A,B), while branching is severely decreased in hypoplastic Yap mutant kidneys at P0 (C,D). Slides were counterstained with DAPI. (E) Calbindin staining was used to quantify branching morphogenesis in control (black columns) and Yap mutant (white columns) at E14.5. Scale bars represent 500 μm. (TIF)

Figure S5 Cell polarity appears normal in Yap and Cdc42 mutant. Immunostaining for Par3 and E-cadherin (A–C, P0), Ezrin (D,E, P0), LTL, and Ecadherin (F,G, P0) and PKC (H–K, E15.5) reveals no defects in cell polarity in early nephrons. Scale bars represent 50 μm. (TIF)

Figure S6 Quantification of Six2 positive cells. Quantification of progenitor cells number using Six2 antibody, at E14.5 reveals a slight but insignificant reduction in Yap mutants compared to controls. (TIF)

Figure S7 Yap deletion impacts nephrogenesis independently of both Wnt/β-catenin and Notch signaling pathways. (A,B) ISH analysis shows normal expression pattern of Wnt9b in both genotypes. (C–P) Staining for known Wnt/β-catenin targets - Ptn2g7 (C,D), Cldcn2 (E,F), Lef1 (G,H), Fig8 (I,J), Wnt4 (K,L), Pax8 (M,N) and Lim1 (O,P) – reveals normal expression in control and mutant kidneys. (Q–X) Yap reveals no effect of Yap deletion on expression of components of the Notch pathway – Notch2 (Q,R), Jag1 (S,T), Hes1 (U,V), Hes3 (W,X). All staining performed at E14.5. Scale bars represent 100 μm. (TIF)

Figure S8 Haploinsufficiency for β-catenin does not alter the YapCM−/- phenotype. (A–C) PAS staining of P0 control, YapCM−/− and Six2;CreTGC/+ Yaplox/lox β-catenin(KO)lox/lox. (D–F) LTL/Calbindin staining of P0 control, YapCM−/− and Six2;CreTGC/+ Yaplox/lox β-catenin(KO)lox/lox. Scale bars represent 1 mm [A–C], 200 μm [D–F]. (TIF)

Figure S9 Changes in gene expression in YapCM−/− kidneys. (A–E) In situ hybridization reveals changes in Uncx4.1 and Soxhd1 in Yap mutants compared to wild-type (E14.5). (G, K) In situ hybridization shows increased levels of expression of Ret (H, G) and Raldh3 (J,K) in UB tips and trunk respectively in E14.5 YapCM−/− kidneys. (C,F,L) Graphical representation of the microarray data of control (black columns) and Yap mutant (white columns). *p<0.05; **p<0.001. Scale bars represent 200 μm. (TIF)

Figure S10 Loss of Cdc42 phenocopies loss of Yap. (A,B) PAS staining of E14.5 wild-type and Cdc42CM−/− kidneys showing the presence of condensing mesenchymal cells (arrows) but dramatic loss of CM-derived epithelial structures (arrowheads). Immunostaining analysis for Six2 (C,D), Sall1 (E,F) and WT1 (G,H) at E14.5 shows presence of CM cells in both genotypes (arrows). E-cadherin and Calbindin were used to visualize the UB compartment. (I,J) Immunostaining at E14.5 shows normal expression of Pax2 in the UB, but decreased expression in the Cdc42-deficient CM cells (arrows). (K,L) In situ hybridization at E14.5 reveals decrease in Uncx4.1 in Cdc42CM−/− mutant. Scale bars represent 100 μm. (TIF)

Figure S11 Lower magnification of Yap localization in MEFs and validation of Cdc42 knock down. Control (A–A’,) and Cre infected (B–B’). Cdc42CMlox/lox MEFs stained with Yap antibody and doubly counterstained with phalloidin and Hoechst 33342. (C) Western-blot analysis using Cdc42 antibody reveals loss of Cdc42 protein in the Cre-infected Cdc42lox/lox MEFs versus control MEFs. Loading control assessed by using α-Tubulin antibody. (TIF)

Figure S12 Sall1 and Mst1/2 removal have minor effects on kidney development. PAS staining (A,B) and NCAM-Pax2 staining (C,D) of P0 control (Pax3;CreERT2, Mst1/2flox/flox, Mst2flox/flox) and Pax3;CreERT2, Mst1flox/flox, Mst2flox/flox showing normal histology. (E–F) Phospho-Yap staining of P0 control and Pax3;CreERT2, Mst1flox/flox, Mst2flox/flox showing comparable phospho-Yap staining in both genotypes. (G–H) PAS staining of E18.5 kidneys from wild-type and Sall1−/− animals. (I,J) E18.5 kidneys stained for proximal tubule markers (LTL) and Pax2. Scale bars represent 100 μm. (TIF)

Figure S13 No change in stromal markers gene expression in Yap mutant. (A,B) ISH analysis reveals similar expression pattern of Raldh2 in both genotype. (C,D) Antibody staining for Foxd1 shows similar expression in both genotypes. E-cadherin was used to visualize the UB compartment. Scale bars represent 100 μm. (TIF)

Figure S14 Loss of N-Wasp leads to hypoplasia and loss of glomeruli and proximal tubules. (A–C) PAS staining (P0) of wild-type and N-WaspCM−/−/ kidneys showing hypoplasia in mutants. (D,E) Sections of P0 kidneys processed for Podocin analysis shows a strong decrease in glomeruli formation in N-WaspCM−/−/ mutants. Average count and standard deviation from four controls and four mutants are shown in F. ***p<0.0001. (G–I) Sections of P0 kidneys processed for LTL and Calbindin staining shows reduced proximal tubule formation in N-WaspCM−/−/ mutant kidneys. Scale bars represent 100 μm (A–E), 200 μm (G–I). (TIF)

Figure S15 Generation of Yap and Taz flox allele. (A) Yap flox allele was generated by inserting LoxP sites for Cre-mediated excision flanking exons 2. (B) The Taz flox allele was generated by inserting LoxP sites for Cre-mediated excision flanking exons 2. (C) Western-blot analysis using Taz antibodies reveals absence of Taz protein in the TazCM−/−/ kidneys. (TIF)

Table S1 Candidates genes of microarrays on E13.5 YapCM−/−/ mutant kidneys compared to Yaplox/lox controls. All 24 genes were assayed by ISH and/or antibody staining on E14.5 wild-type and YapCM−/−/ kidneys. (PDF)

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Author Contributions

Conceived and designed the experiments: AR HM. Performed the experiments: AR RPS AG. Analyzed the data: AR RPS MB-L HM. Contributed reagents/materials/analysis tools: CC D-SL JW TP. Wrote the paper: AR HM.
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