# Cdc42 regulates epithelial cell polarity and cytoskeletal function in kidney tubule development

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**Summary**

The Rho GTPase Cdc42 regulates key signaling pathways required for multiple cell functions, including maintenance of shape, polarity, proliferation, migration, differentiation, and morphogenesis. Although previous studies have shown that Cdc42 is required for proper epithelial development and maintenance, its exact molecular function in kidney development is not well understood. In this study, we define the specific role of Cdc42 during murine kidney epithelial tubulogenesis by deleting it selectively at the initiation of ureteric bud or metanephric mesenchyme development. Deletion in either lineage results in abnormal tubulogenesis, with profound defects in polarity, lumen formation, and the actin cytoskeleton. Ultimately, these defects lead to renal failure. Additionally, in vitro analysis of Cdc42-null collecting duct cells shows that Cdc42 controls these processes by regulating the polarity Par complex (Par3/Par6/aPKC/Cdc42) and the cytoskeletal proteins N-Wasp and ezrin. Thus, we conclude that the principal role of Cdc42 in ureteric bud and metanephric mesenchyme development is to regulate epithelial cell polarity and the actin cytoskeleton.

## Introduction

Mammalian kidney development begins when the ureteric bud (UB) invades the surrounding metanephric mesenchyme (MM) (Carroll and Das, 2013; Costantini and Kopan, 2010; Dressler, 2006). The UB is induced to undergo iterative rounds of branching morphogenesis and gives rise to the collecting system, while the MM forms the nephrons that consist of the glomeruli and renal tubules. Reciprocal signaling between the UB and the MM is required for UB branching morphogenesis and MM development.

Cdc42, a member of the Rho GTPases, plays a critical role in regulating multiple cell functions including cell shape, polarity, proliferation, invasion, migration and differentiation (Melendez et al., 2011). Cdc42 mediates these functions by controlling cytoskeletal dynamics due to its ability to interact with the Wiskott-Aldrich syndrome protein (Wasp) and p21-activated kinases (PAKs). Wasp regulates actin polymerization and filopodia formation by direct interactions with both profilin and actin, and PAKs alter the activity of the critical actin-binding protein cofilin (Melendez et al., 2011). In addition, Cdc42 regulates epithelial cell polarity by forming complexes with the PAR protein family, atypical protein kinase C (PKC) and cadherins (Goldstein and Macara, 2007).

Both in vitro and in vivo evidence suggest a critical role for Cdc42 in kidney development. Cdc42 is required by Madin-Darby canine kidney (MDCK) cells to form tubules in 3-dimensional culture by regulating the formation of the apical plasma membrane domain and tight junctions as well as primary ciliogenesis (Choi et al., 2013; Kim et al., 2007; Martin-Belmonte et al., 2007; Rodriguez-Fraticelli et al., 2010a; Rogers et al., 2003; Zhang et al., 2001; Zuo et al., 2011). Two studies have shown that Cdc42 is essential for normal kidney development and function. In the first, Cdc42 deletion from renal tubular epithelia with the Ksp-cre (which causes cre-mediated recombination in developing nephrons and the renal collecting system) resulted in lethal cystic kidney disease that was attributed to abnormalities in ciliogenesis (Choi et al., 2013). In a second study, Cdc42 was deleted in the nephron

progenitors using Six2-cre. These mice formed hypoplastic kidneys with a reduced nephrogenic zone and small papillae (Reginensi et al., 2013). This phenotype was attributed to defects in localization of the transcriptional activator Yap. While both studies demonstrate roles for Cdc42 in kidney development, their proposed mechanisms of action for Cdc42 are different and out of keeping with its principal function as a master regulator of the actin cytoskeleton and a mediator of epithelial cell polarity.

There is little known about how apical basal polarity is established during renal tubulogenesis. Afadin, an F-actin binding and nectin adaptor protein, was shown to be essential for the timely initiation of apical basal polarity during epithelialization of developing nephrons (Yang et al., 2013). In addition, this adaptor protein was essential for the formation of a continuous apical surface and lumen formation (Yang et al., 2013). At the molecular level, absence of afadin in developing nephrons resulted in delayed lumen formation and reduced ability to correctly localize members of the Par complex (Par3/Par6/aPKC/Cdc42) to the forming epithelia, suggesting that defects in Par complex function and Cdc42 activation might be the underlying defect. Afadin is necessary for Cdc42 activation in vitro (Kawakatsu et al., 2002; Kurita et al., 2013; Mandai et al., 2013; Nakanishi and Takai, 2004), suggesting that the principal mechanisms whereby Cdc42 regulates tubulogenesis in the kidney might be by altering the actin cytoskeleton and apical basal polarity of the epithelial cells.

Given the discrepant findings of Cdc42 function in renal epithelial tubulogenesis compared to its demonstrated function in other epithelia, and the recent findings suggesting that afadin may regulate Cdc42 and renal epithelial cell polarity, we investigated the role of Cdc42 in the developing kidney, focusing on its role in establishing apical-basal polarity and in cytoskeletal architecture. We used $Cdc42^{flox/flox}$ mice crossed with two different cre deletor strains; Six2-cre and Hox-B7-cre. Six2-cre deleted Cdc42 in the cap mesenchyme of the MM and was used in one of the prior studies (Reginensi et al., 2013). Hoxb7–cre deleted Cdc42 in the UB, which gives rise to the collecting system starting at E10.5. We show that Cdc42 is

required for both UB and MM development as the Hoxb7-cre:Cdc42$^{flox/flox}$ mice develop a severe branching morphogenesis defect, and Six2-cre:Cdc42$^{flox/flox}$ mice are unable to form nephrons. Both mice have serious abnormalities in epithelial cell polarization at all developmental stages examined. In particular, the Six2-cre:Cdc42$^{flox/flox}$ kidney phenotype is highly reminiscent of the afadin mutants with disruption of Par complex formation and discontinuous lumens. Using Cdc42-null collecting duct (CD) cells, we showed that absence of Cdc42 caused profound adhesion, migration, and polarization defects, as well as marked reduction in N-Wasp and ezrin activation. Thus, we conclude that a primary function of Cdc42 in the formation of kidney tubules is to promote epithelial cell polarization through Par complex formation and to regulate cytoskeletal architecture via a Cdc42/N-Wasp/ezrin signaling pathway.

## Results

**Cdc42 is required for ureteric bud development.** We crossed Hoxb7-cre mice, which express cre recombinase in the Wolffian duct and UB from E10.5 onward, with Cdc42$^{flox/flox}$ mice to define the role of Cdc42 in the developing UB. The mice were born in the predicted Mendelian ratio; however, all the Hoxb7-cre; Cdc42$^{flox/flox}$ mice died by four weeks of age. Cdc42 deletion was confirmed in mutant mice by performing immunohistochemistry on E15 embryos (Fig. 1A-B) and immunoblotting on isolated papillae of newborn Hoxb7-cre;Cdc42$^{flox/flox}$ with an antibody directed at Cdc42 (Fig. 1C). The kidneys of the Hoxb7-cre;Cdc42$^{flox/flox}$ mice were much smaller than their wild type controls and had histological features of end stage renal disease characterized by severe destruction of the cortex and medulla (Fig1.D-I). When Hoxb7-cre;Cdc42$^{flox/flox}$ mice were sacrificed prior to end-stage renal failure; there was relative preservation of the cortex compared to the severe tubular abnormalities in the medulla. The markedly decreased number of tubules found within the renal papilla of the dysmorphic dysplastic Hoxb7-cre;Cdc42$^{flox/flox}$ kidneys were consistent with a major branching morphogenesis defect (Fig. 1G). Many lumens of the CDs were not

patent (marked by arrows in Fig. 1H) and there were also areas of marked interstitial fibrosis between the dilated cortical CDs (marked by * in Fig 1I).

We next defined when the branching defect in the mice began by performing histological studies of kidneys at various embryonic stages. As early as E12.5 there were fewer UB structures (marked by arrows) and less MM induction in mutants compared to wildtype (Fig. 2A-B). This became more evident over time. The mutant UB was very poorly formed at E15.5 (Fig. 2C-D) and E18.5 (2E-F) with fewer nephrons at both these time points. The branching defect was confirmed by calbindin staining at E18.5 (Fig. 2 G-H). To define why the kidneys of the Hoxb7-cre;Cdc42$^{flox/flox}$ mice were hypoplastic, we quantified proliferation and apoptosis in the kidney. Interestingly there was no statistically difference in proliferation of the cytokeratin-positive UB cells in the Hoxb7-cre;Cdc42$^{flox/flox}$ kidneys as defined by phosphohistone-H3 staining cells at E13.5, E15.5 or E18.5 (Fig. 2I-J, M). In contrast, when we assessed proliferation of Six2 positive cells at E18.5, there was a 60% decrease in the Hoxb7-cre;Cdc42$^{flox/flox}$ kidneys (Fig. 2I-J, N) and this difference was also seen in newborn mice (data not shown). There was no difference in apoptosis rates between the two genotypes at any time points (data not shown). Thus, deletion of Cdc42 in the developing UB caused a severe UB branching defect and secondarily impaired cell proliferation within the MM, which presumably contributed to the reduced kidney size.

**Cdc42 is required for metanephric mesenchyme development.** Cdc42 was deleted from the MM by crossing the Cdc42$^{flox/flox}$ with the Six2-cre mouse. Six2 is expressed in the cap mesenchyme and this Cre line deletes floxed genes from all components of the developing nephron extending from the glomerular epithelium to the connecting segment (Kobayashi et al., 2008). We verified that Cdc42 was deleted in the developing MM by performing immunoblotting and immunohistochemistry on P1 Cdc42$^{flox/flox}$ and Six2-cre;Cdc42$^{flox/flox}$ mice (Fig. 3A-C). Staining revealed clearly decreased Cdc42 expression in the cortical kidney tissue of Six2-cre;Cdc42$^{flox/flox}$ mice. Because all the Six2-cre;Cdc42$^{flox/flox}$ mice died within 24 hours of birth, we defined when the kidney abnormalities first became evident. Although the

kidneys appeared normal at E12.5 (Fig. 3D, E), decreased MM development was evident by E15 (data not shown). This defect was clearly obvious at E18.5 (Fig 3F, G) and P1 where there were markedly decreased numbers of renal vesicles, comma-shaped and S-shaped bodies (Fig 3H, I). The few glomeruli that were seen in the mutant mice were hypoplastic and dysplastic. Mutant pups died within a few hours of birth, presumably due to lack of filtration through the hypoplastic and dysplastic nephrons. Lotus Tetragonolobus Lectin (LTL) positive proximal tubules were greatly reduced in mutants although they were evident and abundant in wildtype controls at E18.5 (Fig. 3J, K). There was 40% less proliferation of Six2 positive cells as defined by phosphohistone-H3 staining cells in the MM of Six2-cre;Cdc42$^{flox/flox}$ kidneys at E15.5 when compared to Cdc42$^{flox/flox}$ kidneys (Fig. 3 L-N) and there were no differences in apoptosis between the two genotypes (data not shown). Thus, removing Cdc42 in the MM resulted in severe hypoplasia and dysplasia of the developing renal vesicles and a profound proliferation defect.

**Cdc42 is required for normal polarization and differentiation of the UB.** One of the features of the kidneys of the Hoxb7-cre;Cdc42$^{flox/flox}$ mice was obstruction of the tubular lumens (Fig. 1), which suggested polarity defects in the tubular epithelial cells. As a major function of Cdc42 is to regulate epithelial polarity, we defined whether there were abnormalities in expression of the highly conserved polarity proteins that form the Par complex found in the apical domain of polarized epithelial cells. To do this, we performed immunostaining of two key components of the complex, Par3 and atypical protein kinase C (aPKC) in Hoxb7-cre;Cdc42$^{flox/flox}$ mice. There was decreased apical staining of aPKC and Par3 in all cells derived from the UB (E-cadherin, cytokeratin-double positive epithelia) in Hoxb7-cre;Cdc42$^{flox/flox}$ mice at E15.5 (Fig 4A-D) and E18.5 (Sup 1 A-D), suggesting a polarity defect in the developing UB that persists over time. Consistent with these apical polarity abnormalities, there was markedly decreased expression of the tight junction protein ZO-1 in the Hoxb7-cre; Cdc42$^{flox/flox}$ mice (Sup 1 E, F). The cells within the developing UB of the Hoxb7-cre:Cdc42$^{flox/flox}$ mice also had severe defects of terminal differentiation as they

did not express the apical water channel aquaporin-2 (Aqp2), while the Cdc42$^{flox/flox}$ mice did (Fig. Sup1 E, F). To verify these terminal differentiation defects of the collecting duct were generalized, we investigated whether there was an altered expression level of the vacuolar-type H ATPase, which is expressed exclusively in the intercalated cells of the collecting duct. This protein was not detectable in the Hoxb7-cre:Cdc42$^{flox/flox}$ mice (Fig. Sup 2A-B). Finally, we also measured the number of cilia present in the Hoxb7-cre:Cdc42$^{flox/flox}$ mice as Cdc42 has been shown to be required for cilia production (Zuo et al., 2011). There were 50% fewer cilia in Hoxb7-cre:Cdc42$^{flox/flox}$ mice compared to Cdc42$^{flox/flox}$ mice (Fig. Sup 2C-E). Taken together, these data indicate that the polarized epithelial cells in the UB of the Hoxb7-cre:Cdc42$^{flox/flox}$ mice have a profound polarity and terminal differentiation defect, which is associated with decreased primary cilia. These cellular abnormalities likely cause intraluminal tubular obstruction that subsequently result in dilated tubules and obstruction of the kidney.

**Defects in MM epithelial cell polarity results in abnormal renal vesicle formation.** During nephrogenesis, mesenchymal nephron progenitors compact to form a pretubular aggregate of cells that subsequently form a polarized sphere of epithelia called the renal vesicle (RV), which contains a central lumen. In pretubular aggregates, neural cell adhesion molecule (NCAM) marks the plasma membrane, but becomes restricted to basolateral membranes as a renal vesicle lumen is formed. Pretubular aggregates have little aPKC, and Par-3 is found in dispersed short segments of plasma membrane, called “pre-apical domains” (Yang et al., 2013). As the renal vesicle lumen forms, both aPKC and Par3 become abundant at the apical surface, with Par-3 becoming redistributed to apical, lateral junctions shortly thereafter (Yang et al., 2013). We found that while Six2-cre;Cdc42$^{flox/flox}$ kidneys do form NCAM positive pre-tubular aggregates, these structures do not progress to a structure with a patent lumen by E15.5 (Fig. 5) and E18.5 (Supp. Fig. 3). aPKC is greatly reduced or absent from many cells while NCAM remains relatively uniform in its plasma membrane localization. Similar to what is observed for aPKC, many structures at this stage lack Par-3, or contain only small foci of Par-3. As demonstrated previously (Reginensi et al.,

2013), deletion of Cdc42 in Six2 positive cells resulted in marginal reduction in total YAP expression in the MM (Fig. Sup4 A-B, arrow). The localization of the phosphorylated form of YAP however remained unchanged (Fig. Sup 4C, D). Thus, Cdc42 is needed for the timely initiation of lumen formation during renal vesicle formation and it is associated with reduced YAP expression.

We also examined the next stage of nephrogenesis, when the renal vesicle elongates into a primordial tubule called the s-shaped body. In the $Cdc42^{flox/flox}$ kidneys, the elongated s-shaped tubule fuses with the tubule of the UB and forms a continuous lumen throughout the s-shaped body and UB at this stage. By contrast, in Six2-cre;$Cdc42^{flox/flox}$ kidneys there is evidence of lumen formation, but the lumens are short and discontinuous despite the apparent fusion of the s-shaped body to the UB (Fig. 6). The lumens of mutants remain discontinuous even in the later stages of the s-shaped body, suggesting it is not simply a delay in their ability to inter-connect. From these studies we conclude that Cdc42 is required not only for timely lumen initiation, but also for continuous lumen formation during kidney morphogenesis.

**CD cells lacking Cdc42 are unable to form tubules and have polarity defects.** Our in vivo findings suggested that deleting Cdc42 resulted in severe abnormalities in tubulogenesis in both the MM and the UB due at least in part to polarity defects. To determine the mechanism of this deficiency, we isolated CD cells from $Cdc42^{flox/flox}$ mice and deleted Cdc42 in vitro using adenoviral-mediated delivery of a plasmid encoding Cre recombinase. We completed serial dilutions on cell populations and identified cells that did not express Cdc42 by immunoblotting (Fig. 7A). We initially performed experiments on at least 3 different populations to show that the results obtained were not due to clonal selection (data not shown). When $Cdc42^{flox/flox}$ CD cells were placed in 3-dimensional collagen-I/matrigel gels, they were unable to form tubules and few cells were observed under these conditions (Fig. 7B, C). Mechanistically, CD cells need to be able to adhere,

spread and migrate through extracellular matrix to form tubules. We therefore defined which of these processes were defective in the Cdc42$^{-/-}$ CD cells. We identified a severe adhesion defect in the Cdc42$^{-/-}$ CD cells on collagen I, laminin-111 (not shown) and -511 (not shown) and fibronectin (Fig 7F) although the cells had normal expression of integrin β1, α1, α2, α3, α5 and α6 subunits (data not shown). Thus, deleting Cdc42 altered cell adhesion of CD cells on multiple extracellular matrix (ECM) components, suggesting this adhesion defect was not integrin specific. Consistent with the adhesion defects, these cells had a profound haptotactic migration defect on collagen 1, laminin-111 (not shown) and -511 (not shown) and fibronectin (Fig. 7G). They also had a major spreading defect on collagen 1 (Fig. 7 D, E), laminin-111 and -511 and fibronectin (data not shown). Due to the small number of cells seen in the Cdc42$^{-/-}$ CD cell tubulogenesis assay, we performed proliferation assays of these cells on multiple ECMs including collagen 1 and fibronectin under low serum conditions. In agreement with the results seen in vivo and in the in vitro 3D assays, there was a marked decrease in Cdc42$^{-/-}$ CD cell proliferation that is independent of the ECM (Fig. 7H). Thus, Cdc42 deletion from CD cells results in a severe CD cell tubulogenesis defect due to decreased adhesion, spreading, migration and proliferation.

Mechanistically, Cdc42 regulates multiple cellular processes, including polarization, actin cytoskeletal organization and cell signaling. Based on our in vivo and in vitro data, we initially investigated whether the Cdc42$^{-/-}$ CD cells had a polarization defect by growing them on transwells and performing immunostaining for the adherens junction protein, E-cadherin, and the tight junction protein, ZO-1. Much less of both these proteins localized to the cell-cell junctions of the Cdc42$^{-/-}$ CD cells when compared to Cdc42$^{fl/fl}$ CD cells (Fig 7I-L). We next employed biochemical techniques to determine whether there was decreased expression or mislocalization of E-cadherin, ZO-1 and the apical proteins Par3 and aPKC in the Cdc42$^{-/-}$ CD cells. There was marked decrease of E-cadherin, ZO-1 and Par3 and a moderate decrease of aPKC in the Cdc42$^{-/-}$ CD cells (Fig. 7M, N). When we analyzed the relative amounts of these proteins in triton soluble and insoluble cell fractions, there was less E-

cadherin, ZO-1, aPKC and Par3 in the triton insoluble (membranous) fraction of $Cdc42^{-/-}$ CD cell lysates relative to $Cdc42^{fl/fl}$ (control) cell lysates (Fig. 7O-Q). Taken together, these data demonstrate a polarity defect in $Cdc42^{-/-}$ CD cells, which is consistent with our in vivo findings.

**$Cdc42^{-/-}$ regulates the actin cytoskeleton in the collecting system of the kidney.** The severe adhesion, migration and spreading defect manifested by the $Cdc42^{-/-}$ CD cells suggested they have abnormal integrin-dependent signaling. When we investigated this possibility, no differences between $Cdc42^{f/f}$ and $Cdc42^{-/-}$ CD cells were found when we assessed activation of well recognized signaling proteins such as Akt or ERK that act downstream of integrins using re-plating assays on collagen 1 or laminin-111 and -511 (data not shown). Cdc42 is also reported to be a key regulator of cytoskeletal proteins such as N-Wasp which activates the Arp2/3 complex and ezrin, which binds N-Wasp (Albiges-Rizo et al., 2009). When we defined how Cdc42 regulated the activation of these proteins in response to glial derived neurotropic factor (GDNF), a principal growth factor responsible for UB branching morphogenesis, phosphorylation of both these proteins was severely decreased in $Cdc42^{-/-}$ compared to $Cdc42^{fl/fl}$ CD cells, despite the same amount of activation of the Ret receptor (Fig. 8A). These data suggest that the severe adhesion, spreading and migration defects observed in the $Cdc42^{-/-}$ CD cells, are due at least in part to the requirement of Cdc42 for N-Wasp and Ezrin activation and this signaling defect is not due to decreased Ret activation.

We next examined whether an actin cytoskeleton defect was present in vivo by staining the developing UB at E15.5 with rhodamine-phalloidin. Interestingly there was no obvious structural abnormality in the subapical distribution of actin in the Hoxb7-cre:$Cdc42^{flox/flox}$ mice (Fig 8 B, C). The subapical actin also appeared normal in distribution in the Six2-cre;$Cdc42^{flox/flox}$ mice except in the areas in which there was no discernible lumen (Fig 6). As a lack of obvious structural defects does not necessarily indicate normal actin cytoskeleton function, we examined the distribution of ezrin. In $Cdc42^{flox/flox}$ mice ezrin staining was clearly

expressed in the apex of the lumen of the UB, however this staining was decreased and diffuse at the apical surface of CDs in the Hoxb7-cre:Cdc42$^{flox/flox}$ mice (Fig. 8 D, E), which is consistent with our in vitro data. Together, these data demonstrate that Cdc42 is critical for apical actin cytoskeletal function in developing collecting ducts and N-Wasp/ezrin signaling of CD cells in vitro.

**Discussion**

Cdc42 has diverse cellular functions and plays a role in development of many organs. We show the absence of Cdc42 in the UB causes severe branching abnormalities as well as profound polarity and cytoskeletal defects that cause malformed and obstructed tubular lumens. Similarly, deleting Cdc42 from the developing MM produced severe abnormalities in epithelial polarity and lumen formation resulting in failed renal vesicle and s-shaped body establishment. We confirmed the requirement for Cdc42 for tubule formation in vitro utilizing Cdc42-null CD cells which also showed severe adhesion, migration, proliferation, polarization and cytoskeletal defects. Thus we conclude that the principal role of Cdc42 in UB and MM development is to regulate epithelial cell polarity and actin cytoskeleton function.

It has previously been shown that deletion of Cdc42 in the distal tubule of the kidney using the Ksp-cre induced fatal kidney failure, however the few surviving mice developed cystogenesis associated with decreased ciliogenesis and interstitial fibrosis (Choi et al., 2013). Most of the characterization of these mice was performed within the cysts where increased cell proliferation and apoptosis associated with increased MAPK pathway activation was noted. No epithelial cell polarization defects were observed, as measured by E-cadherin or gp135 localization. The discrepancies between this and our studies could be attributed to the fact that Hoxb7-cre deletes Cdc42 at the initiation of UB branching morphogenesis in the newly forming bud tips at E10.5 (Yu et al., 2002). In contrast, Ksp-cre deletes at E12.5 in the stalk region of the UB, and from E15.5 onward, in more distal

segments of the nephron (Karner et al., 2009; Shao et al., 2002). It is not clear whether the cysts observed in the Ksp-cre mutants are in the CD or the distal tubule. It is interesting to note that similar to Choi et al (Choi et al., 2013), we did observe a decrease in cilia number in mutant collecting ducts. This cellular phenotype was not as penetrant as defects in polarity and differentiation suggesting it may be a secondary manifestation of Cdc42 deletion.

Our morphological results of the Six2-cre;Cdc42$^{flox/flox}$ in the developing MM are very similar to those reported by Reginensi and colleagues (Reginensi et al., 2013), in that the kidneys were severely hypoplastic with almost no functional nephrons. They concluded that the principal mechanism whereby Cdc42 regulated MM development was by altering the cytoskeleton via an N-WASP-dependent mechanism which altered nuclear localization of YAP. Further, they did not comment on a major proliferation or polarity defect and they reported no changes in Par3 or aPKC localization. Indeed we did find that deletion of Cdc42 in Six2 positive cells resulted in marginal reduction in total YAP expression in the MM (Fig. Sup4 A-B, arrow), although the expression of the phosphorylated form of YAP appeared unchanged (Fig. Sup 4C, D). Our study identified several other major cellular defects, including abnormalities in epithelial cell polarity and proliferation, tubule lumen formation, and actin cytoskeletal function. We postulate these are the primary causes of the defects in terminal differentiation of both the nephron and the collecting duct epithelia. Our study is consistent with the findings in other branching organs such as the pancreas (Kesavan et al., 2009) and lung (Wan et al., 2013) where epithelial deletion of Cdc42 was shown to be required for branching, epithelial cell proliferation, tubule formation, normal apical polarity and actin cytoskeleton formation as well as terminal differentiation and cell-fate specification. In addition, hepatocytes from Cdc42-null mice were unable to proliferate in response to hepatectomy (Yuan et al., 2009) and deleting Cdc42 from the colon resulted in a mild defect in apical junction orientation and impaired intestinal epithelium polarity (Melendez et al., 2013), clearly demonstrating a role for Cdc42 in epithelial cell proliferation and polarity.

Our observations in both the UB and MM are very similar to those demonstrated in pancreatic duct formation, where Cdc42 plays a cell-autonomous role in microlumen formation and in generation of apical polarity and tight junction formation (Kesavan et al., 2009). Therefore, we propose that the tubular lumen and cell differentiation lesions in the Hoxb7-cre:Cdc42$^{flox/flox}$ and Six2-cre:Cdc42$^{flox/flox}$ mice are primarily caused by abnormalities in epithelial cell polarity due to defects in Cdc42-dependent aPKC activation through Par6 (Goldstein and Macara, 2007) and abnormal apical actin cytoskeletal function caused by abnormal N-Wasp activation as described in other systems (Rohatgi et al., 2000; Rohatgi et al., 1999). The Cdc42 Six2-cre mice have a phenotype that is similar to mice lacking afadin: both are defined by their polarity defects and discontinuous lumen formation as evidenced by a decrease in Par3 staining in the pretubular aggregates/renal vesicles and the absence of a discrete lumen at the renal vesicle stage (Yang et al., 2013). The Six2-cre:Cdc42$^{flox/flox}$ mutants do not pass through a true renal vesicle stage and small discontinuous lumens develop in s-shaped bodies resulting in a persistent defect in luminal continuity. There are increased levels of both Par3 and aPKC in s-shaped bodies of Cdc42 Six2-cre mutants compared with earlier stages. The mechanisms responsible for this “recovery” of the localization of Par complex components are unknown, but it is likely that partially redundant signaling pathways compensate in the absence of Cdc42. Similar to the Cdc42 mutants, afadin mutants also have defects in the actin cytoskeleton as they are unable to recruit the actin subapical ring to form apical surfaces. In addition, afadin mutants fail to form Par complexes. Together, these results suggest that Cdc42 and afadin may act in a similar pathway to promote polarization and lumen formation. Based on in vitro data from others (Kawakatsu et al., 2002; Kurita et al., 2013; Mandai et al., 2013; Nakanishi and Takai, 2004), we speculate that afadin may act upstream of Cdc42 in the signaling pathway, however this still needs to be verified.

We used Cdc42$^{-/-}$ CD cells to demonstrate that Cdc42 is required for in vitro 3-dimensional tubulogenesis as well as CD cell adhesion, migration, and proliferation on all extracellular

matrices. Similar to our in vivo findings, these cells also have a major abnormality in epithelial cell polarity with respect to formation of adherens and tight junctions as well as the Par3/Par6/aPKC complex. Our results are similar to those demonstrating a role for Cdc42 in polarized mammary epithelial cell migration and branching where Cdc42 was either overexpressed or knocked down in vitro (Duan et al., 2010) and overexpressed in the mouse mammary gland in vivo (Bray et al., 2013). In the kidney tubular epithelium the functional characteristics of Cdc42 have been explored in the well-characterized polarized MDCK cells. Utilizing over expression and knockdown techniques, Cdc42 was shown to be required for lumen formation of tubules because it mediates exocytosis of large intracellular vacuoles that become the lumen and it also binds to the Par3/Par6/aPKC complex, which regulates tight junction formation and epithelial cell polarity (Martin-Belmonte et al., 2007; Martin-Belmonte and Mostov, 2007; Martin-Belmonte et al., 2008; Rodriguez-Fraticelli et al., 2010b). In addition Cdc42 was shown to play a role in localizing the exocyst to the primary cilium of MDCK cells (Zhang et al., 2001; Zuo et al., 2011). The effects of deleting Cdc42 on MDCK cell tubulogenesis are much less severe than those seen in the $Cdc42^{-/-}$ CD cells, which never progress to the cyst stage in 3D-culture. These data suggest very distinct roles for Cdc42 in tubulogenesis and lumen formation in CD and MDCK cells and that Cdc42 plays multiple roles in tubule formation in different cell types.

We demonstrate that the principal signaling defect observed in $Cdc42^{-/-}$ CD cells is their inability to activate N-Wasp and ezrin in response to growth factors. There was also a major defect of apical ezrin expression and localization in CDs in vivo. Together these data suggest that local recruitment of activated Cdc42 and its downstream effector, N-Wasp, can trigger actin polymerization in the apical domain of the epithelial cell (Castellano et al., 1999; Nakamura et al., 2000). This is consistent with the well-defined role of Cdc42 in localizing the Par3/Par6/aPKC complex to the apical part of a polarized epithelial cell (Joberty et al., 2000; Welchman et al., 2007).

In conclusion, we and others have shown that Cdc42 is required for normal tubulogenesis in UB and MM development of the kidney (Choi et al., 2013; Reginensi et al., 2013). While the morphological observations are similar in all these studies, the mechanisms proposed differ. We demonstrate both in vivo and in vitro that the predominant role of Cdc42 in renal tubulogenesis, as in other branching organs like the lung (Wan et al., 2013) and the pancreas (Kesavan et al., 2009), is to regulate epithelial cell proliferation, polarity and the actin skeleton. Thus, in keeping with its pleiotropic effects, Cdc42 regulates epithelial cell tubulogenesis by multiple mechanisms. This emphasizes the importance of studying its cell autonomous functions in different situations in epithelial cell biology.

## Materials and Methods

**Mice:** All experiments were approved by the Vanderbilt University Institutional Animal Use and Care Committee. $Cdc42^{flox/flox}$ mice which were previously described (Wu et al., 2006) were crossed with the Hoxb7Cre mice (generous gift of Dr A. McMahon) (Yu et al., 2002) or Six2-cre mice (generous gift of Dr A. McMahon) (Kobayashi et al., 2008). Mice were a F4-F6 generation toward the C57/B6 background. Aged-matched littermates $Cdc42^{flox/flox}$ mice were used as controls.

**Morphologic and immunofluorescence analysis:** For morphological and immunohistochemical analysis, kidneys were removed at different stages of development and fixed in 4% formaldehyde and embedded in paraffin. Paraffin tissue sections were stained with either hematoxylin and eosin or Periodic Acid Schiff's (PAS) for morphological evaluation by light microscopy.

Immunofluorescence was performed as previously described (Yang et al., 2013). In brief, fixed sections (4% PFA for 2 hours) were permeabilized with 0.3% Triton X-100/PBS (PBST) and blocked with 10% BSA in PBST. Sections were incubated with primary antibodies overnight (4°C), then with fluorophore-conjugated secondary antibodies and mounted with

Vecatshield (Vector Laboratories). Antibodies used were Par3 (1:150, Millipore, 07-330), aPKCζ (1:400, Santa Cruz Biotechnology, C-20) and phalloidin 647 and 488 (1:200, Invitrogen, 42008A), YAP and pYAP (1:500, Cell signaling 4912, 4911, tyramide amplification), V-ATPase (1:500, Santa Cruz Biotechnology, 20943), cytokeratin (1:500, Sigma Aldrich, C2562), LTL (1:500, Vector labs, B1325), NCAM (1:500, no antigen retrieval), E-cadherin (1:300, Invitrogen), DBA (1:500, Vector Labs, B1035), Ezrin (1:500, Upstate 07-130 ), ZO1 (Santa Cruz Biotechnology, 33725), Aqp2 (Sigma Aldrich, A7310), Acetylated alpha tubulin (Cell Signaling, 5335P), Six2 (Proteintech, 11562-1) . Secondary antibodies were purchased from Jackson ImmunoResearch and AlexaFluor antibodies from Invitrogen.

Cell Proliferation in vivo. Kidney sections obtained at different embryonic stages were subjected to immunohistochemical analysis using primary antibodies against phospho histone H3 (pHH3, 1:2000, Sigma Aldrich, tyramide amplification), Six2 (1:500, Proteintech) and Cytokeratin (1:500, Sigma Aldrich). More than 250 Six2 or CK positive cells were counted from each kidney at each stage and the percentage of pHH3 co-positive cells were calculated. For each stage and genotype a minimum of 4 different embryos were analyzed.

**Generation of Cdc42$^{-/-}$ cells**: CD cells were isolated from Cdc42 $^{fl/fl}$ mice following the methodology described by Husted et al (Husted et al., 1988) and Cdc42 was deleted by infecting the cells with an adenocre virus in vitro. Deletion of Cdc42 was verified by immunoblotting for Cdc42. Clonal Cdc42 null cell lines were generated and similarity in their behavior was verified.

**Tubulogenesis Assay.** CD cells were grown in collagen/matrigel gels as previously described (Chen et al., 2004). CD cells ($5 \times 10^3$) were seeded into the gels, which were overlaid with 100 µl of medium and allowed to grow for 5 to 7 days. The gels were stained with rhodamine-phalloidin, (R415, 1:2000, Molecular Probes) and the tubules were photographed using a Zeiss Axio 510 confocal microscope (400×).

**Cell Spreading:** Cells were plated onto slides coated with Collagen I (10 µg/ml) for 2 hours after which they were fixed, permeabilized, exposed to rhodamine phalloidin (1:5000) and visualized under a fluorescence microscope.

**Cell adhesion and migration assays:** Cell adhesion and migration assays on different ECM components were performed as described (Chen et al., 2004). Four independent experiments were performed in triplicate.

**Cell Proliferation:** Proliferation assays were performed as previously described (Chen et al., 2004). Briefly, 5 x $10^3$cells were plated on different ECM components in 96-well plates and maintained in DMEM (10% FBS). Twelve hours later the cells were switched to DMEM (2% FBS) for 24 hours and then pulsed with 1 µCi/well [3H] thymidine (PerkinElmer Life Sciences). Twenty-four hours later, the cells were solubilized and radioactivity was measured using a scintillation counter.

**Cell Polarity:** Cells were grown on transwells consisting of polyvinylpyrolidone-free polycarbonate filters with 0.4µm pores. After reaching confluency, cells were fixed in 4% formaldehyde and incubated with anti-ZO-1 (1:200; BD Transduction Laboratories) or E-cadherin (1:1000; BD Transduction Laboratories) antibodies followed by the appropriate FITC-conjugated secondary antibody. Chamber slides were mounted and viewed using a confocal microscope.

**Immunoblotting:** The effect of glial-derived neurotropic factor (GDNF) on CD cells were examined as previously described (Zhang et al., 2009). In brief, cells were trypsinized into serum-free DMEM and then plated on collagen I (10 µg/ml) for 45 minutes. GDNF (10 ng/ml) was added to the medium and the cells were lysed at different time points following growth factor stimulation.

For analysis on kidney tissues, the cortices or medullas were removed and lysed with T-Per buffer (ThermoScientific). Lysates were clarified by centrifugation and 30 µg total protein

was electrophoresed onto a 10% SDS-PAGE and subsequently transferred to PVDF membranes. Membranes were blocked in 5% milk with TBS Tween and then incubated with the different primary antibodies followed by the appropriate HRP-conjugated secondary antibodies.

Immunoreactive bands were identified using enhanced chemiluminescence according to the manufacturer's instructions. Cdc42(2466,1:1000), ezrin (3142, 1:1000), phospho ezrin (3144, 1:1000) and phospho-Ret (3221, 1:1000) antibodies were purchased from Cell Signaling; PKC zeta antibody from Upstate (17264,1:1000), ParD3A (sc-98509,1:1000) antibody from Santa Cruz Biotechnology N-Wasp (wp2001,1:1000) and pN-Wasp (wp2601,1:1000) from ECM Biosciences, ZO-1 (33-9100,1:1000) from Life Technologies, E-cadherin (610181,1:2000) from BD Biosciences

**Cell Fractionation**: Triton-insoluble (actin-rich) and Triton-soluble fractions of CD cells were prepared as described previously (Elias et al., 2014). Briefly, cells were incubated for 5 min with lysis buffer-CS (50 mm Tris/HCl, pH 7.4, 1.0% Triton X-100, 5 mm EGTA, and 10 µg/ml protease inhibitor mixture). Cell lysates were centrifuged at 15,600 × g for 5 min at 4 °C to sediment the high density actin-rich fraction. The pellet was suspended in 200 µl of lysis buffer D (0.3% SDS in 20 mm Tris/HCl buffer, pH 7.4, and 10 µg/ml protease inhibitor mixture).

**Statistics:** The Student's t test was used for comparisons between two groups, and analysis of variance using Sigma Stat software was used for statistical differences between multiple groups. $p< 0.05$ was considered statistically significant.

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## Figures

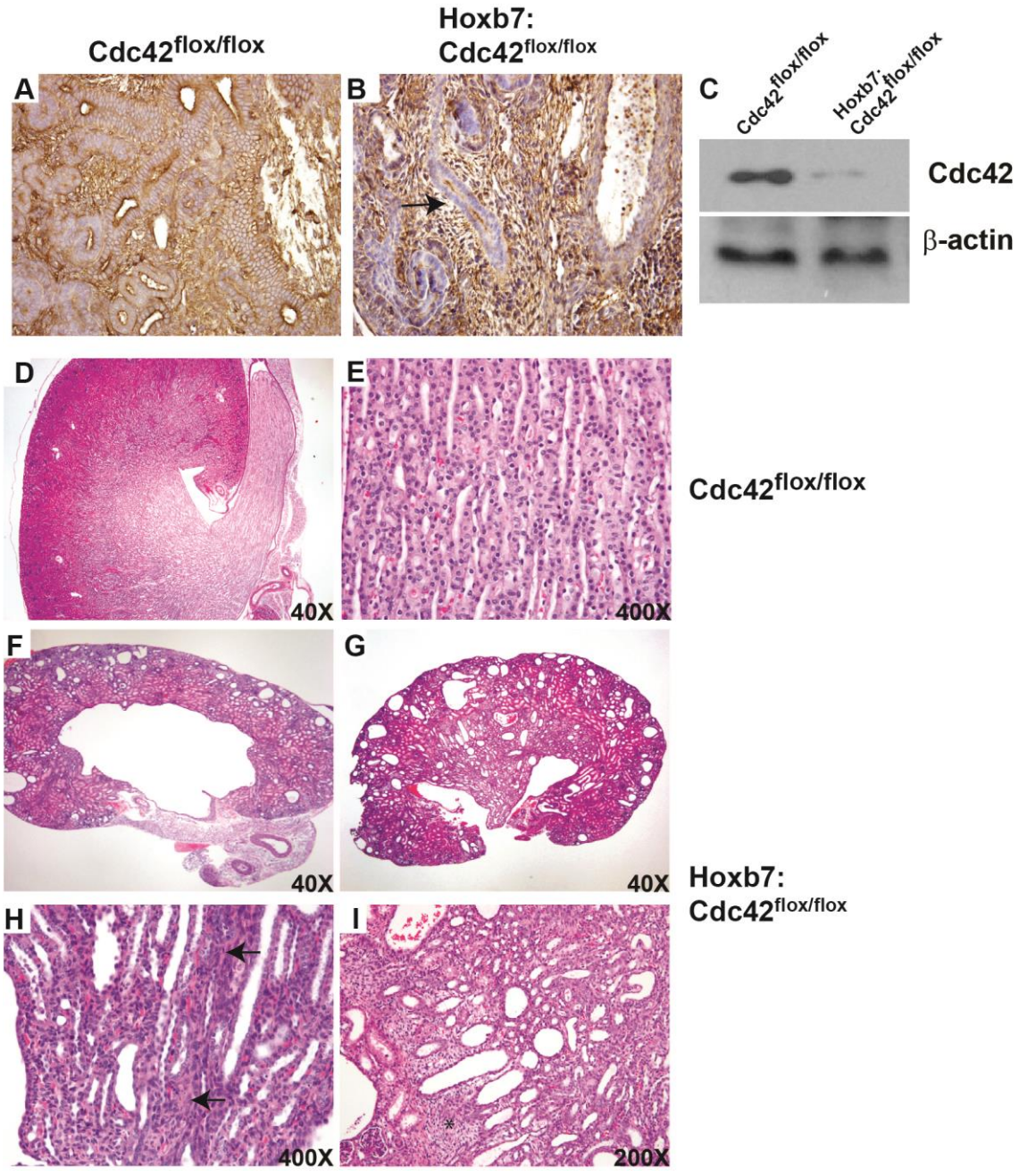


**Figure 1. Hoxb7-cre:Cdc42$^{flox/flox}$ mice develop end-stage renal failure due to defective branching morphogenesis and intraluminal obstruction in the CDs.** (A-C) Sections of

E15.5 mouse kidneys from Cdc42$^{flox/flox}$ and Hoxb7-cre:Cdc42$^{flox/flox}$ was stained with antibodies directed against Cdc42. No Cdc42 was visualized in the developing UB (arrow) of Hoxb7-cre:Cdc42$^{flox/flox}$ mice (A-B). Deletion of Cdc42 in the Hoxb7-cre:Cdc42$^{flox/flox}$ mice were confirmed by immunoblotting the kidney papilla of newborn mice with an anti-mouse Cdc42 antibody (C). (D-I) Microscopy of hematoxylin and eosin (H&E) stained kidney slides showing destruction of the medulla and corticomedullary junction in Hoxb7-cre:Cdc42$^{flox/flox}$ but not in the Cdc42$^{flox/flox}$ kidneys. Total destruction of the medulla due to hydronephrosis in Hoxb7-cre:Cdc42$^{flox/flox}$ mice is present just prior to death at 4 weeks of age (F), while at 2 weeks of age the kidneys are severely hypoplastic and dysplastic with dilated tubules found within the cortex and medulla and evidence of focal tubular cysts (G). High power images (H) show the medulla where the lumens of the CDs are filled with cells (arrows) and the tubules are dilated. Dilated tubules and interstitial fibrosis (*) are seen in the cortex (I)

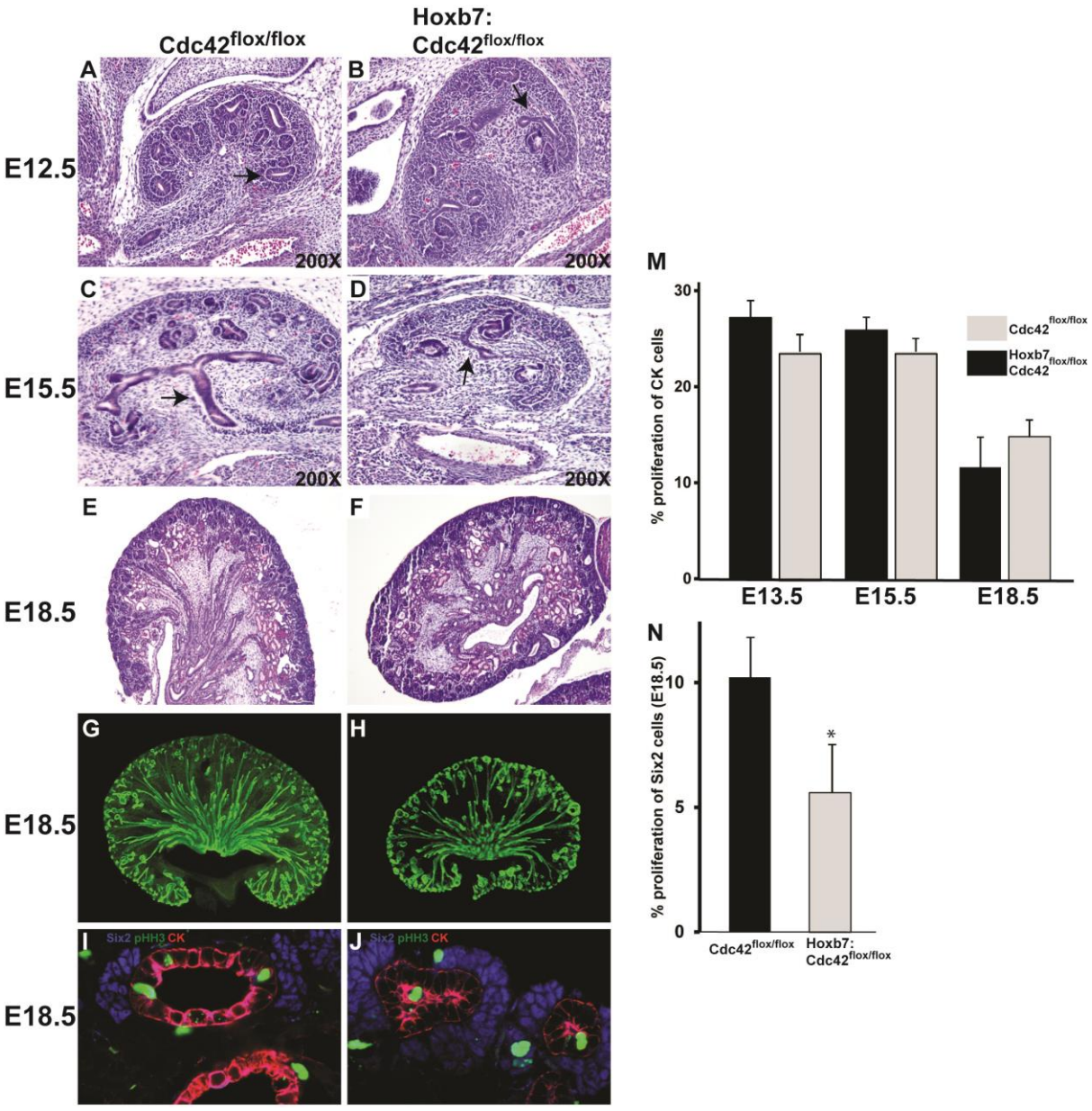


**Figure 2. Hoxb7-cre:Cdc42$^{flox/flox}$ mice have a severe branching morphogenesis defect and impaired MM induction.** (A-H) Kidneys were isolated from embryos of the Cdc42$^{flox/flox}$ and the Hoxb7-cre;Cdc42$^{flox/flox}$ mice at E12.5 (A, B), E15.5, (C, D) and E18.5 (E-J). A marked UB branching morphogenesis defect (arrows) and decreased MM induction was noted at each of these time points. Calbindin staining demonstrated a decrease in UB

structures at E18.5 in the Hoxb7-cre;Cdc42$^{flox/flox}$ when compared to the Cdc42$^{flox/flox}$ mice (G-H). Phosphohistone-H3 staining was performed on cytokeratin positive cells (marking UB structures, red) and Six2 positive cells (marking MM structures, blue ) (I-J). The percentage of proliferation at different time points in the UB (M) and MM (N) was quantified. Differences between Cdc42$^{flox/flox}$ and the Hoxb7-cre;Cdc42$^{flox/flox}$ (*) were significant in the Six 2 positive cells ($P<0.05$).

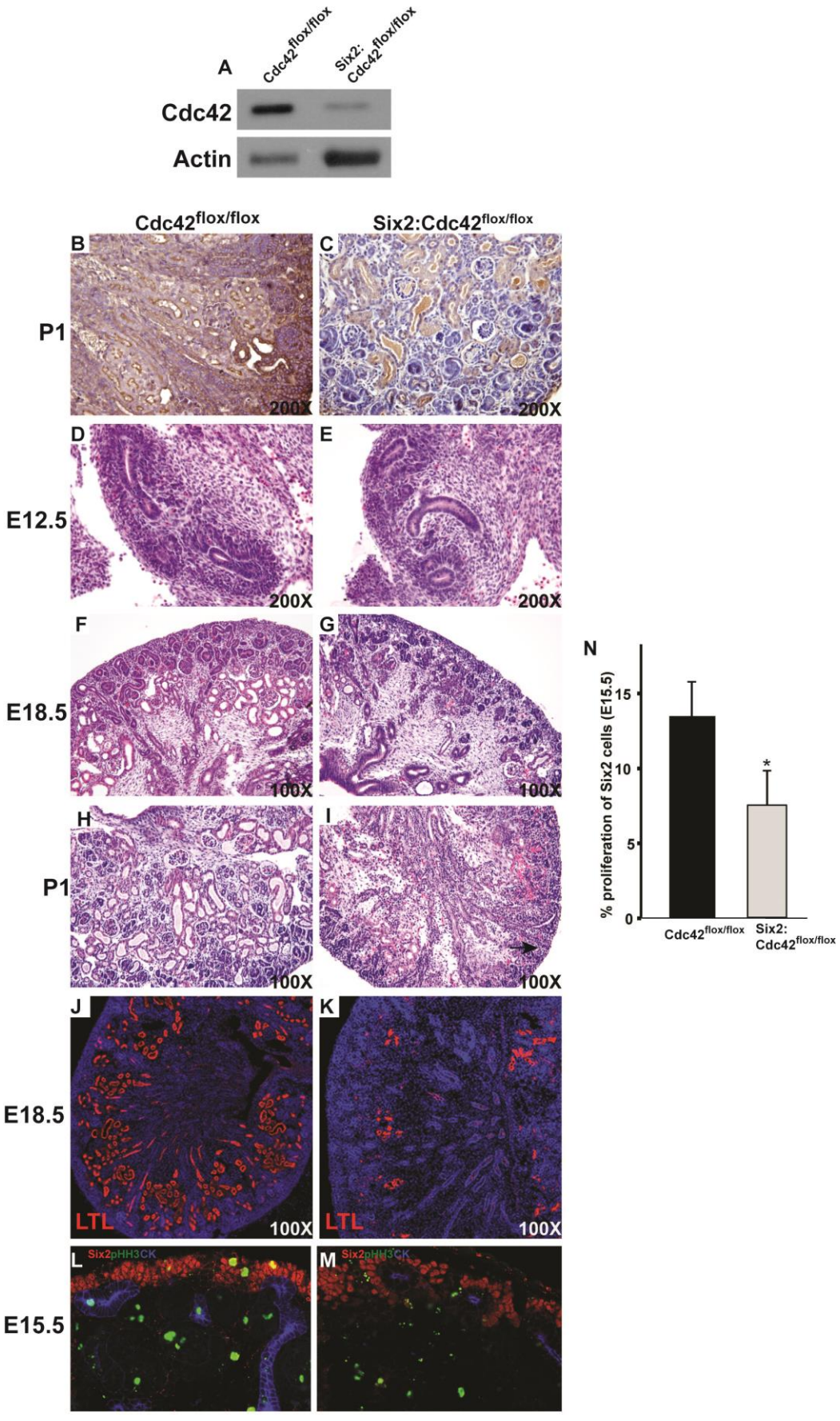

**Figure 3. Six2-cre;Cdc42$^{flox/flox}$ mice have a defect in metanephric mesenchyme development.** (A) Deletion of Cdc42 in the Six2-cre;Cdc42$^{flox/flox}$ mice was confirmed by immunoblotting the kidney cortex of newborn mice with an anti-mouse Cdc42 antibody. (B-C) No staining with an anti-Cdc42 antibody was seen in the MM of newborn Six2-cre;Cdc42$^{flox/flox}$ mice. (D-E) No UB branching defect was seen in the E12.5 Six2-cre;Cdc42$^{flox/flox}$ mice. (F-I) Diminished MM development was seen in E18.5 Six2-cre:Cdc42$^{flox/flox}$ mice (F-G), which was even more severe at P1 (H-I) with almost no nephrons formed (arrow) in the MM. (J-K) The diminished MM development was verified in E18.5 Six2-cre:Cdc42$^{flox/flox}$ kidneys as decreased lotus tetragonolobus lectin (LTL), a marker of proximal tubules, staining. (L-N) Phosphohistone-H3 staining was performed on Six2 positive cells of E15.5 kidneys from Cdc42$^{flox/flox}$ (L-M) and the Six2-cre;Cdc42$^{flox/flox}$ mice and quantified (N). Differences between Cdc42$^{flox/flox}$ and the Six2-cre;Cdc42$^{flox/flox}$ (*) were significant ($P<0.01$).

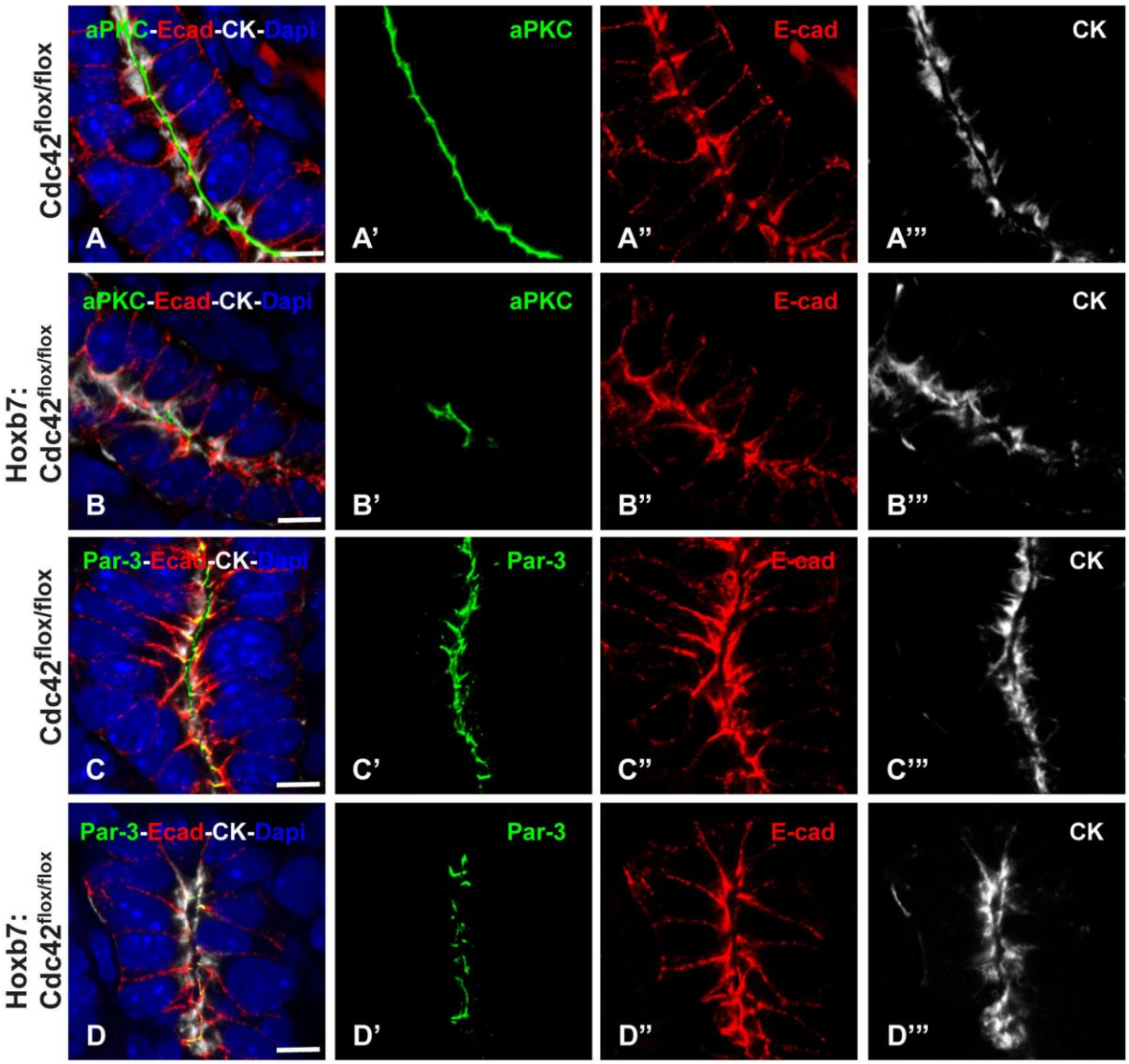


**Figure 4. : Localization of apical proteins in the collecting ducts of wildtype and Cdc42 mutant kidneys at E15.5.** Immunostaining of E15.5 Cdc42 $^{flox/flox}$ (A, A', A'', C, C', C'') and Hoxb7-cre:Cdc42 $^{flox/flox}$ (B, B', B'', D, D', D'') kidneys stained with antibodies to aPKC (green in A, A', B, B'), Par-3 (green in C, C', D, D'), E-cadherin (red in A'', B'', C'' and D'') and cytokeratin (CK) (white in in A''', B''', C''' and D'''). A, B, C and D are merged panels while individual panels are marked by ', '' or '''. All sections have been stained with DAPI (blue in A, B, C, D) to show the nuclei. Scale bar: 20microns.

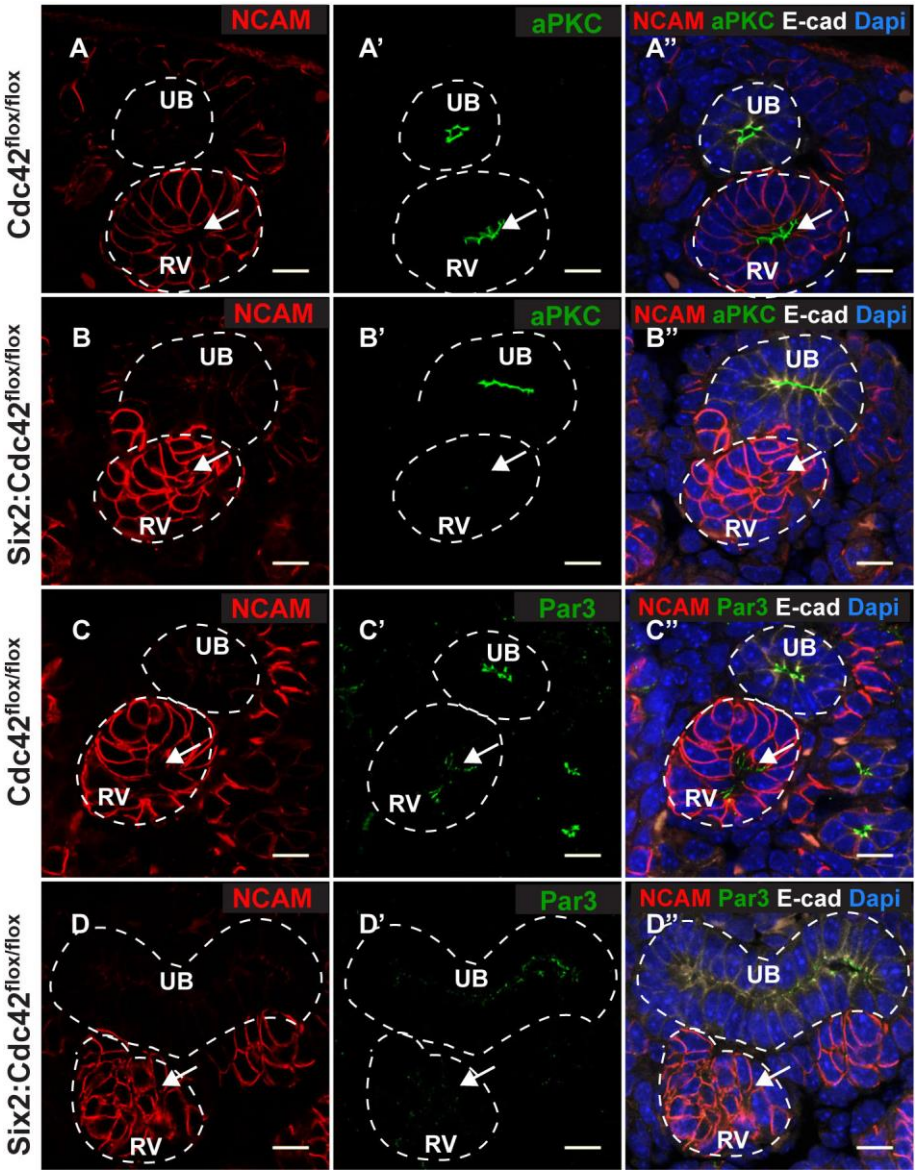


**Figure 5. Cdc42 is essential for timely lumen formation in developing nephrons.** Immunostaining of E15.5 kidneys from Cdc42$^{flox/flox}$ (A-A''; C-C'') and Six2-cre:Cdc42$^{flox/flox}$ (B-B'', D-D'') with NCAM (red), aPKC or Par-3 (green), E-cadherin (white), and DAPI (blue) as indicated. Controls show luminal clearing of nuclei with apical aPKC, basolateral NCAM, and apical-lateral Par-3. In contrast, mutants have no evidence of a central clearing of nuclei, display relatively uniform NCAM with little/no aPKC. Mutants also have reduced Par-3, with

some mutant PA/RV structures showing none, or small foci of Par-3. Results are representative of sections from at least 2 mice. Similar results were obtained at E18.5 (Supplemental Fig 2). Abbreviations: RV, renal vesicle; PA, pretubular aggregate, UB, ureteric bud. UB and RV/PA are outlined by a white dotted line. Scale bars: 10 μm.

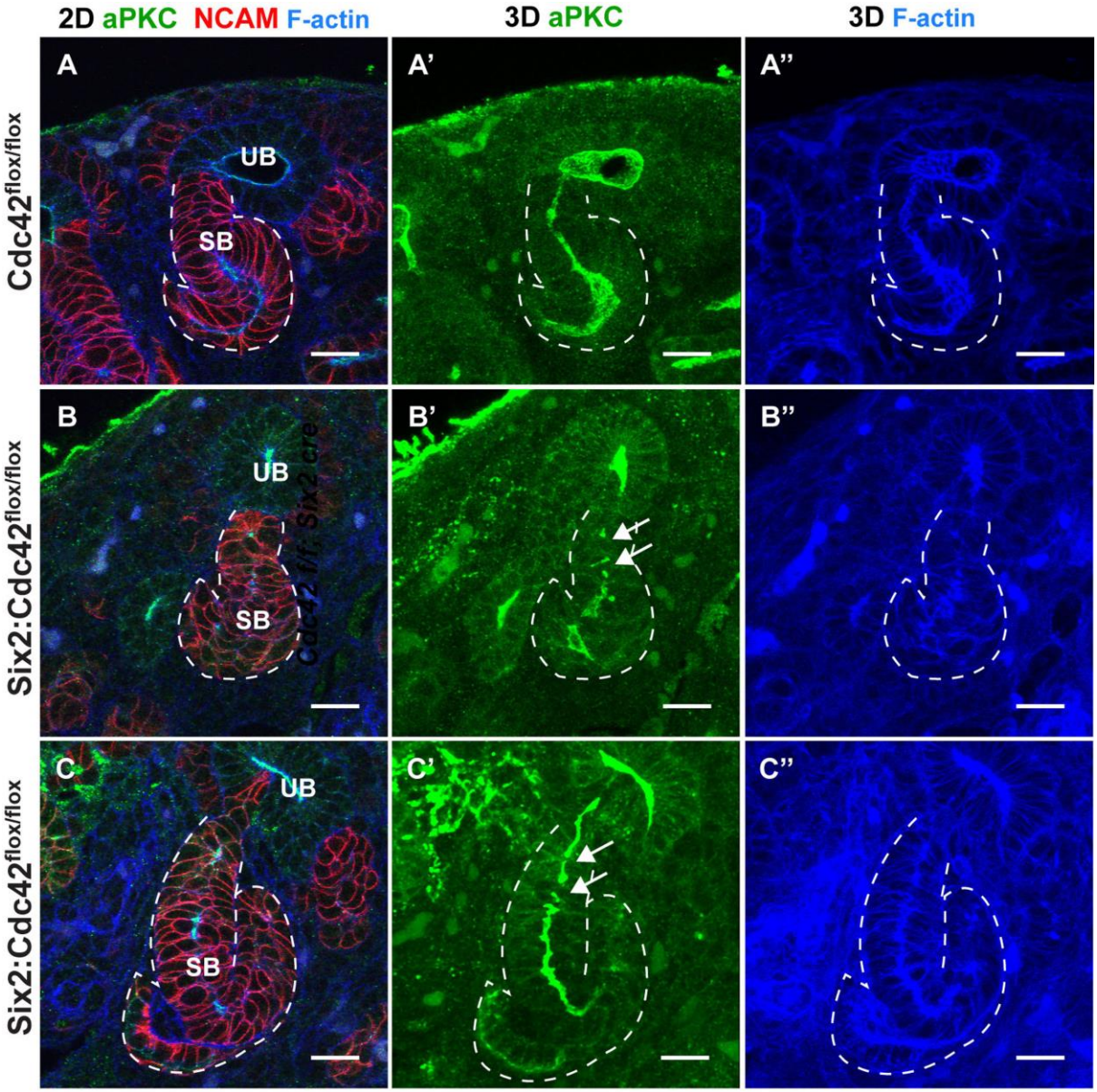


**Figure 6. Cdc42 is required for continuous lumen formation in nephron tubules.** Shown are 2D images and 3-D reconstructions from confocal z-stacks of E15.5 kidneys immunostained with anti-aPKC (green), F-actin (phalloidin, blue), and anti-NCAM (red) as indicated. aPKC marks the apical surface, thereby demarcating the lumen. NCAM delineates basolateral cell membranes of developing nephron tubules. The s-shaped body stage of nephron tubulogenesis is shown in Cdc42$^{flox/flox}$ (A-A'') and Six2-cre;Cdc42$^{flox/flox}$ (B-

C'') mice. Images from A and B show s-shaped bodies (SB) at a similar stage, highlighting the discontinuous SB lumen in mutants (arrow). Images from C show a late s-shaped nephron, illustrating that the discontinuous lumens persist in mutants. Results are representative of sections from at least 2 mice. Abbreviations: SB, s-shaped body; UB, ureteric bud. SB is outlined by a white dotted line. Scale bars: 10 μm.

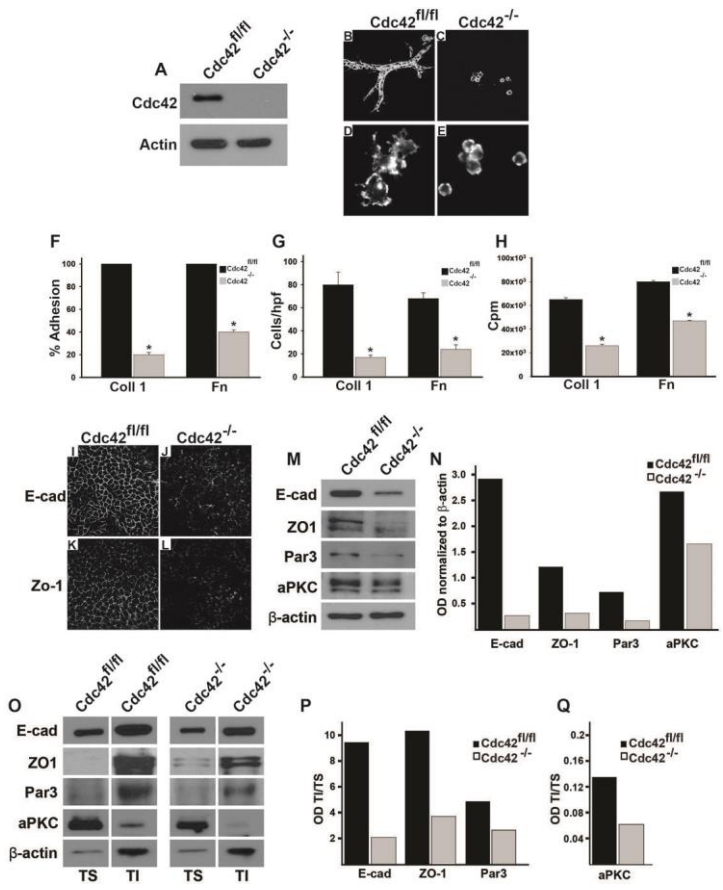


**Figure 7. Deleting Cdc42 from CD cells results in abnormal tubulogenesis, adhesion, migration, proliferation and polarity defects**. (A) Immunoblotting was performed on Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ cells to verify Cdc42 deletion. (B-C) Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells were placed into 3D collagen and Matrigel gels and allowed to undergo tubulogenesis over 7 days in the presence of 5% FBS. They were stained with rhodamine phalloidin and visualized by confocal microscopy. (D-E) Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells were plated on collagen I and allowed to spread for 1 hour, after which they were stained with rhodamine phalloidin. (F) Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells populations were allowed to adhere to collagen I and fibronectin (10ug/ml) and cell adhesion was evaluated 1 hour after plating. Values are the mean ± s.d. of three experiments performed in triplicates. (*) denotes a

statistically significant difference (P<0.01) between Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells (G) Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells were plated on transwells coated with collagen I (10 μg/ml) or fibronectin (10 μg/ml) and migration was evaluated after 4 hours. This was quantified and expressed as cell/high power field (cells/hpf). Values are the mean ± s.d. of three experiments performed in triplicates. (*) denotes a statistically significant difference (P<0.01) between Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells. (H) Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells were plated on collagen I. After 24 hours, cells were treated with 3H-Thymidine and incubated for a further 24 hours. 3H-Thymidine incorporation was then determined and expressed as counts per minute (cpm) as described in the Materials and methods. Values are the mean ± s.d. of three experiments performed in triplicates. (*) indicates a statistically significant difference (P<0.01) between Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells. In F-H, values are mean ± s.d. from three experiments performed in triplicate, and asterisks denote statistically significant differences ($p$<0.01) between Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells. (I-L) Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells were grown to confluence on transwell inserts and immunostained for ZO-1 and E-cadherin. (M-N) Equal amounts of whole cell lysate from Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells were electrophoresed and immunoblotted for E-cadherin, ZO-1, Par3, atypical PKC (aPKC) and β actin. The intensities of a single blot were measured and shown on a graph (N), which is representative of at least three similar experiments. (O-Q) Equal amounts of triton-soluble (TS) and insoluble (TI) fractions from Cdc42$^{flox/flox}$ and Cdc42$^{-/-}$ CD cells were analyzed by Western blot for E-cadherin, ZO-1, Par3, atypical PKC (aPKC) and β actin. The intensities of a single blot were measured and shown graphically (P-Q). This is representative of at least three similar experiments.

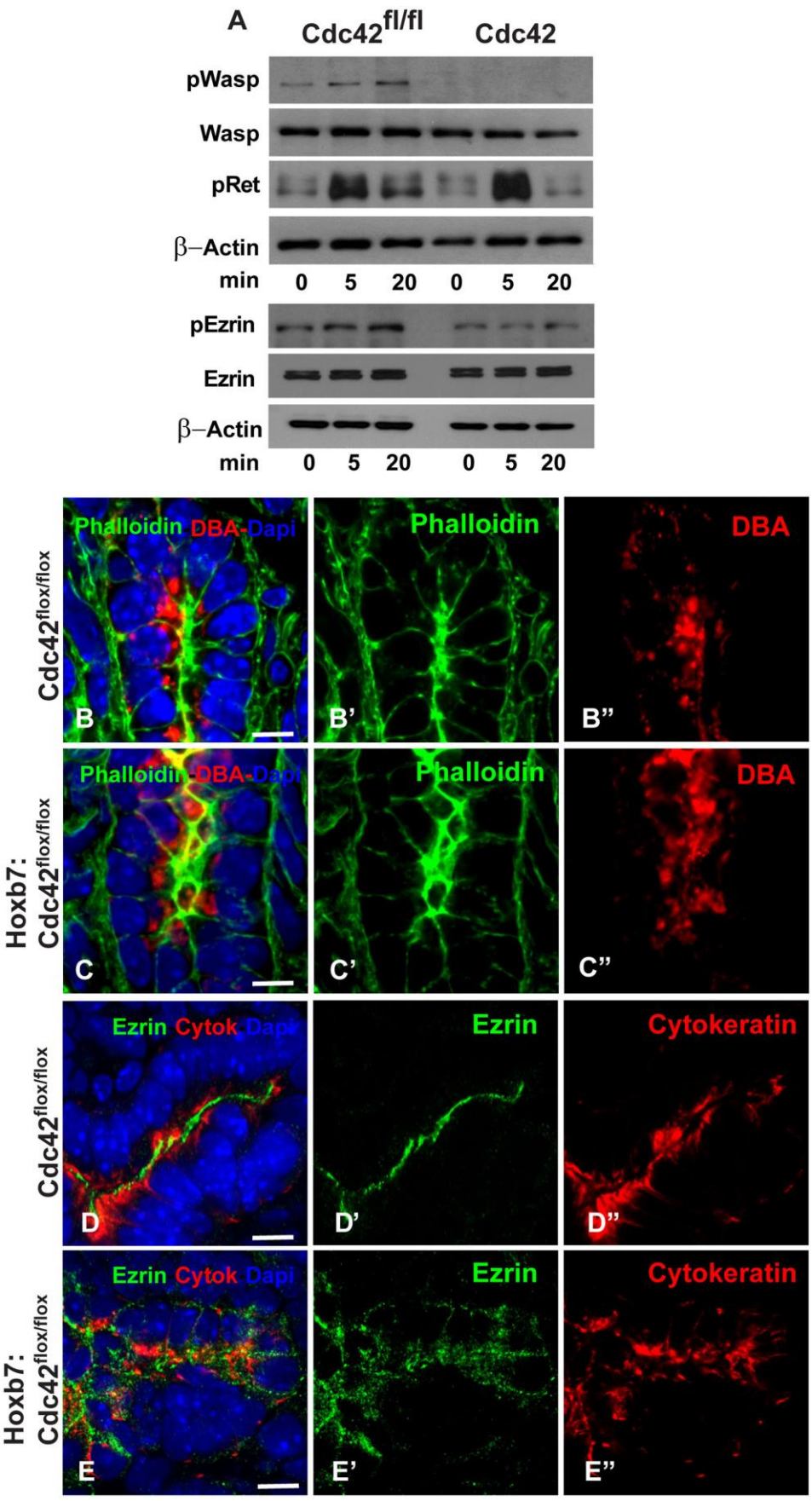

**Figure 8. Deleting Cdc42 from CDs results in cytoskeletal abnormalities.** (A) $Cdc42^{flox/flox}$ and $Cdc42^{-/-}$ CD cells were allowed to adhere to collagen I for 1 h, after which they were treated with GDNF for 5 and 20 minutes. The cells were then lysed and analyzed by Western blotting for levels of phospho-Wasp (pWasp), Wasp, phospho-Ret, phospho-ezrin (pEzrin), ezrin and β-actin. Immunostaining of E15.5 $Cdc42^{flox/flox}$ (B, B', B'', D, D', D'') and Hoxb7-cre:$Cdc42^{flox/flox}$ (C, C', C'', E, E', E'') kidney sections with phalloidin (green in B-C''), the collecting duct marker DBA (red in B-C''), nuclear marker DAPI (blue in B, C, D and E), the sub-apical marker ezrin (green in D-E'') and the collecting duct marker cytokeratin (red in D-E''). B, C, D and E represent merged images while B' and C' are single images for phalloidin, B'' and C'' are single images for DBA, D' and E' are single images for Ezrin and D'' and E'' are single images for cytokeratin. Scale bar 20microns.