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Rap1 regulates lumen continuity via Afadin in renal epithelia

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Abstract

The continuity of a lumen within an epithelial tubule is critical for its function. We previously found that the F-actin binding protein Afadin is required for timely lumen formation and continuity in renal tubules formed from the nephrogenic mesenchyme in mice. Afadin is known effector and interactor of the small GTPase Rap1, and in the current study, we examine the role of Rap1 in nephron tubulogenesis. Here, we demonstrate that Rap1 is required for nascent lumen formation and continuity in cultured 3D epithelial spheroids and *in vivo* in murine renal epithelial tubules derived from the nephrogenic mesenchyme, where its absence ultimately leads to severe morphogenetic defects in the tubules. By contrast, Rap1 is not required for lumen continuity or morphogenesis in renal tubules derived from the ureteric epithelium, which differ in that they form by extension from a pre-existing tubule. We further demonstrate that Rap1 is required for correct localization of Afadin to adherens junctions both *in vitro* and *in vivo*. Together, these results suggest a model in which Rap1 localizes Afadin to junctional complexes, which in turn regulates nascent lumen formation and positioning to ensure continuous tubulogenesis.

SUMMARY STATEMENT

We find that Rap1 is required for central lumen formation in renal epithelia and that this is mediated via correct localization of the F-actin binding protein Afadin.

Graphical Abstract

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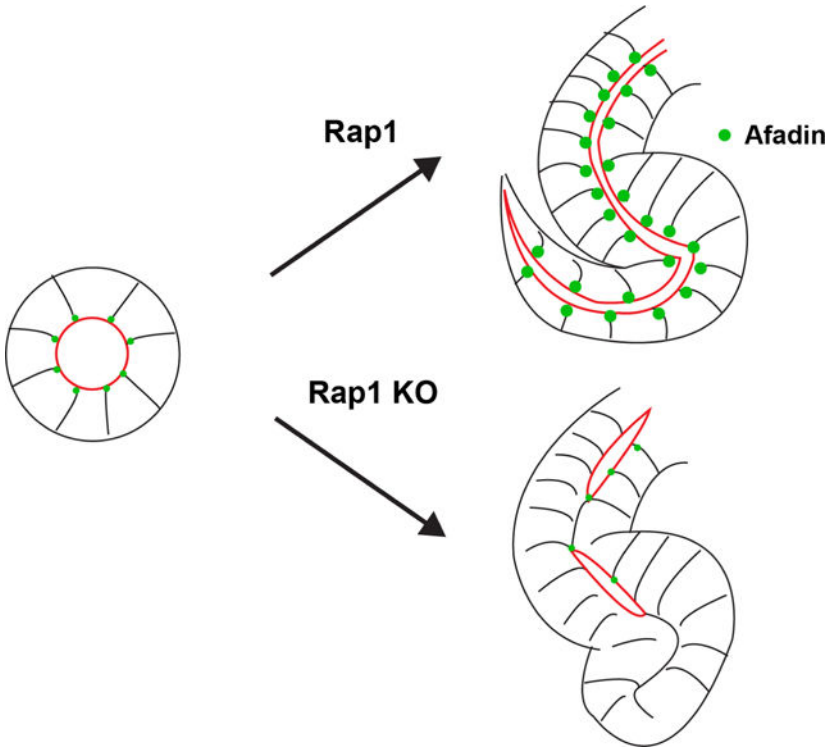
AUTHOR CONTRIBUTIONS

Conceptualization: DKM, CH, LG; Supervision: DKM; Data analysis: CH and LG; Writing of the first draft: DKM, CH. All authors performed experiments, generated figures and reviewed/revised the manuscript.

DISCLOSURES

None.



Keywords

Kidney development; lumen; tubulogenesis; Rap1; Afadin; polarity; nephron; epithelial cells; tubules

INTRODUCTION

In the kidney, a mature nephron contains a polarized epithelial tubule comprised of multiple functionally and structurally distinct segments. During kidney development, nephron tubule formation occurs when the nephrogenic cap mesenchyme undergoes a mesenchyme-to-epithelia transition, forming an epithelial sphere that subsequently elongates into an s-shaped tubule (1, 2). The s-shaped tubule then fuses with a tubule formed by the ureteric bud epithelia. Together, the joined tubules elongate and differentiate to form the entire tubular portion of the nephron.

Lumen continuity in nephron tubules is critical for the excretory function of the kidney. Indeed, disruption of the polarization process and lumen formation leads to renal dysplasia and cystogenesis in mice (3), linking cell polarity and renal disease. However, many of the cellular mechanisms and proteins that help establish apical-basal polarity to create a single continuous lumen remain elusive. Understanding underlying *in vivo* mechanisms is critical for understanding how congenital and acquired defects of tubulogenesis lead to disease.

The assembly and apical positioning of adherens junctions that link neighboring cells to each other and to the actin cytoskeleton is an early step in establishing apical-basal polarity

(4). Afadin, an F-actin binding protein and nectin adaptor, has emerged as a key regulator of the adherens junction (5). In the developing kidney of mice, Afadin is required for both timely formation and positioning of the lumen in tubules, thereby establishing apical-basal polarity and lumen continuity during kidney tubulogenesis (6). It has a similar role in lumen formation and tubulogenesis in the developing pancreas (7). Afadin also controls apical-basal spindle orientation (8–10), a role that is conserved in several cell types and likely participates in generating a central, continuous lumen.

Afadin interacts with many proteins. Specifically, it has been shown to act as an effector to the small GTPases Rap1a and 1b, which bind to the N-terminal Ras-association (RA) domains of Afadin (11, 12). Rap1a and Rap1b are separate genes (13) that encode for proteins differing in only 9 amino acids and are thought to be functionally redundant. Single cell RNA-seq datasets (14) have revealed that both genes are expressed in renal epithelia, and henceforth, Rap1a and Rap1b will collectively be referred to as Rap1. Rap1 cycles between an inactive GDP-bound state mediated by many specific GTPase-activating proteins (GAPs) and an active GTP-bound state mediated by several specific guanine nucleotide exchange factors (GEFs) (15). In addition to Afadin, activated Rap1 binds to many downstream effectors and is known to play critical roles in various cellular processes such as cell-cell/cell-matrix adhesion, morphogenesis and regulation of polarity (15–18). In Drosophila, Rap1 correctly positions Cno, the Afadin homolog, and cadherin-catenin complexes at apical junctions (19–21). In mammalian tissue, loss of Rap1 results in impaired adhesive interactions and polarity in ocular epithelium, resulting in defective lens morphogenesis (22). Rap1 is also required for junctional integrity and the early morphogenesis of endothelial tubes (23, 24).

To understand the role of Rap1 in nephron tubulogenesis and determine if it participates in an Afadin-signaling module, we examined Rap1 function in the developing mouse kidney and in 3D epithelial spheroid culture. Our data show that Rap1 is required *in vivo* for timely lumen formation and continuity in renal epithelial tubules derived from the nephrogenic cap mesenchyme and in 3D epithelial spheroids. These results underscore the role of Rap1 in nascent lumen formation. The Rap1 phenotype is highly similar to that of loss of Afadin (6), and we find that Rap1 is required for correct localization of Afadin to adherens junctions. These results suggest that Rap1 localizes Afadin to adherens junctions in renal tubules, thereby ensuring timely lumen formation and continuity.

MATERIALS AND METHODS

We crossed *Rap1a*$^{f/f}$*;Rap1b*$^{f/f}$ females to *Rap1a*$^{f/+}$*; Rap1b*$^{f/+}$*; HoxB7-cre* males to obtain *Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; HoxB7 cre* mutant mice (25, 26). Similarly crosses were performed with *Six2-cre* (27). Mice were maintained on mixed genetic backgrounds and genotyped by standard PCR. Neonatal kidneys were harvested and fixed for 2 hours in 4% paraformaldehyde (PFA) in PBS. Six-week-old mice were perfused with 4% PFA and their isolated kidneys fixed for 4 hours with 4% PFA. Procedures were performed according to UTSW-IACUC-approved guidelines.

MDCK culture, shRNA-mediated gene silencing and CRISPR gene editing

MDCK type II cells were grown and imaged as previously described (29). Transfection of MDCK cells with mcherry-Rap1 (a gift of Mark Philips, NYU) was performed with Lipofectamine 2000 (Invitrogen) and stable lines were selected. The shRNA oligos were designed against Rap1a and Rap1b using the iRNAi program (http://www.mekentosj.com/science/irnai) using guidelines from the Addgene pLKO.1 protocol (www.addgene.org). The selected shRNA oligos were as follows: Rap1a shRNA1 : CCGGTATGACCCAACGATAGAAGCTCGAGCTTCTATCGTTGGGTCATATTTTTG ; Rap1a shRNA2 : CCGGGATTCCTACAGAAAGCAAGCTCGAGCTTGCTTTCTGTA-GGAATCTTTTTG; Rap1b shRNA1 : CCGGGGGCTTGCATTATAATTACCTCGAGGTAAT-TATAATGCAAGCCCTTTTTG; Rap1b shRNA2 : CCGGCAGGGAGCCACAGTATTTAC-TCGAGTAAATACTGTGGCTCCCTGTTTTTG.

Rap1a/1b shRNAs were cloned into pLKO.1 puromycin and viral transduction of MDCK cells was performed as previously described (30). For CRISPR knockout lines, sgRNA oligonucleotides were designed using the CRISPR Genome Engineering Resources (http://crispr.mit.edu/). The sgRNAs used for this study were as follows: Rap1a sgRNA1 : CACCgCTGGAAGATGAGCGAGTAGT; Rap1a sgRNA2: CACCgCTAGACAGTTCAGTTTGTTC; Rap1b sgRNA1: CACCgGTTCTTG-GCTCAGGAGGCGT; Rap1b sgRNA2: CACCgCTTGAAATCTTGGATACTGC. Double knockout (KO) clones were generated and selected according to Cong and Zhang (31). Cells were verified for loss of Rap1 by western blot.

Immunofluorescence

Kidney sections were permeabilized with 0.3% Triton X-100/PBS (PBST) and blocked with 10% donkey sera/PBST. Antigen retrieval was performed with Trilogy (Cell Marque). MDCK cysts were fixed in 4% PFA for 10 min and then permeabilized with 0.3% PBST and blocked with 0.7% fish skin gelatin in TBS with 0.025% saponin (PFS). Samples were incubated with primary antibodies overnight (4°C), then with fluorophore-conjugated secondary antibodies. Kidney sections were mounted with Prolong Gold (Invitrogen). Confocal imaging was performed on a Zeiss LSM880 confocal microscope. Images were minimally processed and re-sampled to 300 dpi using Adobe Photoshop.

Western blot

MDCK cysts were lysed in ice-cold buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM sodium phosphate (pH 7.2), 100 mM NaCl, 50 mM Tris-HCl, 50 mM NaF, 4 mM Na3VO4, 1 mM PMSF and a protease inhibitor cocktail (Roche). Sample buffer (4X) was added to lysates and they were boiled for 5 min. These lysates were then centrifuged at 17,000*g* for 15 min at 4°C to remove insoluble aggregates, and SDS-PAGE and western blotting were performed.

Antibodies

For immunostaining, primary antibodies were used at 1:100 unless stated otherwise: Afadin (Sigma, A0224), Par6b (SCBT, sc-67392), Podocalyxin (Developmental Studies Hybridoma

Bank (DSHB), 3F2/D8), ZO1 (Millipore, MABT11), Cadherin 6 (a gift of G. Dressler, University of Michigan, MI, USA), Phalloidin 647 (Invitrogen, 42008A), NCAM (DSHB, 5B8); Ecad (Thermo,13–1900), Muc-1(Thermo, MA5–11202). For western blots, we used Rap1 (1:1000, BD, 610195), Afadin (1:1000, Sigma, A0224) and β-actin (1:5000, Abcam, ab8227) antibodies with HRP-conjugated secondary antibodies (Jackson Immunoresearch).

Statistics

In vitro experiments were performed in triplicate and are representative of at least two similar experiments. All data shown are mean ± s.d. Statistical significance was performed using an unpaired, two-tailed Student's *t-test* unless stated otherwise.

RESULTS

Localization of mCherry-Rap1 during lumen formation

To assess if Rap1 has a role in lumenogenesis, we first examined Rap1 localization during lumen formation in early stage spheroids of Madin-Darby Canine Kidney (MDCK) cells. We generated stable MDCK cell lines with mCherry-Rap1a (32) since our tests of several Rap1 antibodies in control and Rap1-depleted spheroids did not validate any antibodies for immunostaining.

In two-cell spheroids, sequential stages of lumen formation can be delineated by the localization of Podocalyxin (Podxl) and Afadin (33). Prior to lumen initiation (Fig. 1A), Podxl localizes to the peripheral surface of the cells and Afadin localizes to the cell-cell interface. As previously observed, Podxl is then internalized in vesicles by each cell (not shown, see ref (30)) which then fuse to form the apical membrane initiation site (AMIS) (Fig. 1B). At the AMIS stage, Afadin becomes restricted to the entire lateral cell-cell junctions (arrows) (Fig. 1B). As the AMIS matures and opens a lumen (Fig. 1C), Podxl remains at the apical surface while Afadin becomes further restricted to apical cell-cell junctions (arrowheads). The mCherry-Rap1a remains distributed to both apical surfaces and apical junctions throughout lumen formation (Fig. 1A–C).

Rap1 is required for normal lumen formation *in vitro* and recruits Afadin to cell-cell junctions

To assess the role of Rap1 in lumen formation, we generated stable double knockdown (DKD) cells and double knockout (DKO) cell lines for Rap1a and Rap1b, which are 95% identical at the amino acid level. We confirmed that these cell lines were depleted or devoid of Rap1a/b (Fig. 2A). The Rap1a/b deficient spheroids (denoted Rap1 DKD and DKO) formed multiple lumens (Fig. 2B,C), similar to the phenotype of spheroids lacking Afadin (33). They also exhibited a delay in initial lumen formation in 2-cell stage spheroids (Fig. 2D), which is similar to spheroids lacking Afadin.

In Rap1 DKD/DKO spheroids, Afadin localization was diffuse in both early (1-day) and 5-day spheroids (Fig. 3A). There was a noticeable loss of Afadin at apical-lateral cell junctions, compared with controls. We quantified this result in 5-day spheroids by assessing the ratio of Afadin to ZO1 intensity at apical-lateral junctions (Fig. 3B). Since total Afadin

levels were unchanged in the Rap1 DKD/DKO cells (Fig. 2A), we concluded that Rap1 regulates Afadin localization, but not levels, in renal epithelia *in vitro*.

Rap1a/b is required for normal lumen formation in the developing nephron, but not ureteric-bud derived epithelial tubules

During kidney development, epithelial tubules of the kidney arise from two embryonic sources, the cap mesenchyme and the ureteric bud, which give rise to nephron tubules and collecting ducts, respectively. These two tubules form by different mechanisms. The cap mesenchyme undergo a mesenchymal-to-epithelial transition to form an epithelial vesicle, with de novo formation of a central lumen called the renal vesicle. The renal vesicle subsequently elongates to form an s-shaped tubule called the s-shaped body. By contrast, the ureteric bud tubule forms as a branch (or extension) from a pre-existing tubule, the nephric duct. Thus, its lumen is an extension of the preexisting lumen of the nephric duct. The lumen from the s-shaped body ultimately connects with the ureteric bud lumen via anastomosis (6).

We first examined the role of Rap1 in ureteric bud tubulogenesis using conditional deletion of Rap1a/b with the *HoxB7-cre* mouse line, which recombines in the ureteric bud epithelia and its derivatives. At E14.5, mutant kidneys had normal sized kidneys with normal appearance (Fig. 4A). Immunofluorescent staining for the lumenal protein mucin-1 (Muc1), which labels the ureteric bud lumen (as well as distal nephron), revealed that double mutants (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; HoxB7-cre*) had normal continuous ureteric lumens enriched in apical Muc1, similar to controls (Fig. 4B).

Next, we investigated its role in nephron tubulogenesis using the *Six2-cre*, which recombines in the cap mesenchyme (27). Mice with conditional deletion of Rap1a/b (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; Six2-cre*) displayed defects in lumen formation, with marked discontinuous lumens evident in s-shaped bodies (Fig. 4C, Fig. S1, Movies 1a, 1b). The lumens were demarcated by Par6 (Pard6b) at the apical surface and NCAM at lateral cell junctions. This phenotype is similar to what is observed with conditional deletion of Afadin with *Six2-cre* (6). Some of the nephron tubules in *Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; Six2-cre* mutant mice also had abnormal morphogenesis, as clearly depicted in Fig. 4C, which displays an s-shaped body with abnormal shape, particularly at its tail (Fig. 4C, arrow).

We performed Chi-square analysis at the neonatal stage and at 4 weeks-of-age for litters born from a cross of *Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$ and *Rap1a*$^{f/+}$*; Rap1b*$^{f/+}$*; Six2-cre* mice. From 17 litters of neonatal mice, there were 12 mutants of 87 total mice, which was not a deviation from the expected Mendelian frequencies ($X^2 = 2.034$, 3 degrees of freedom, $N = 87$, $p = 0.565$). In contrast, the analysis of 17 litters at 4 weeks-of-age showed a clear deviation from expected Mendelian frequencies ($X^2 = 17.61$, 3 degrees of freedom, $N = 99$, $p < 0.005$): there was only 1 mutant of 99 total mice. We observed that most mutant mice die between the ages of P1 and P5. Thus, because of their early demise, we were unable to perform histological and phenotypic analyses on adult mice.

Given the similar phenotype to Afadin mutants, prior data showing that Afadin is an effector of Rap1, and our results in the MDCK spheroids, we next examined Afadin localization in both *Six-cre* and *HoxB7* mutants. Absence of Rap1 from the nephron tubules (*Rap1a*$^{f/f}$*;*

Rap1b$^{f/f}$*; Six2-cre*) caused a reduction in apical lateral Afadin (Fig. 5A). These results underscore the role of Rap1 in nascent lumen formation during nephron formation and suggest that this is mediated through its effect on Afadin localization. Interestingly, even though deletion of *Rap1* from the ureteric tubules (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; HoxB7-cre*) did not cause lumenal defects, Afadin was also clearly reduced at the apical lateral junctions of ureteric epithelia (Fig. 5B).

DISCUSSION

Rap1 and its effector proteins are known to regulate interactions between AJs, polarity determinants, and the cytoskeleton during epithelial cell polarity establishment and maintenance (15–18). However, its role in organ development and tissue architecture, particularly in mammals, is still not well explored. In the current study, we performed *in vitro* and *in vivo* experiments to examine the role of Rap1 in renal epithelia during kidney development. Our results demonstrate that Rap1 is required for normal lumen continuity *in vitro* and in nephron epithelia *in vivo*, but not in ureteric epithelia. These findings provide novel insights into how GTPase signaling drives kidney morphogenesis.

Given its critical role in regulating junctions (34), it is perhaps not surprising that Rap1 would be important during tissue formation. Dynamic remodeling of adherens and tight junctions between cells occurs as cells change shape, proliferate and migrate, and progressively assemble into tissues during organogenesis. Rap1 is intimately involved in these cellular processes. In Drosophila, activity of Rap1 modulates α-catenin-dependent coupling of junctions and the actin cytoskeleton during epithelial invagination that occurs at gastrulation (35). Only a few groups have examined Rap1 functions in forming mammalian embryonic tissues. One study in mice demonstrated that global loss of Rap1 resulted in early lethality of embryos at E10.5, whereas conditional deletion in blood vessels resulted in severe vascular defects and hemorrhage in a portion of the mutant embryos due to loss of cell-cell junction integrity (23). Along those lines, Rap1 was also shown to enhance barrier function in small blood vessels by potentiating vascular endothelial (VE) cadherin junctions (36). We find our work concurs with these previous studies in that Rap1 is required for tissue morphogenesis and regulates localization of the critical cell adhesion adaptor Afadin, which is required for proper lumen formation in the nephron tubules.

In previous work we showed that, like Rap1, Afadin is essential for the proper morphogenesis of a variety of epithelial tissues. Using genetic deletion of Afadin from the nephron epithelium, we showed it is essential for initial polarity establishment and subsequent lumen placement in the renal vesicle and s-shaped body (33). In a separate study, we found that Afadin, which is expressed in nascent lumens of the pancreatic epithelium, is similarly required for continuity of ductal lumens in that organ (7). In both the kidney and pancreas Afadin knockout organs, there is striking mislocalization of the lumens and their associated adherens and tight junctions. These studies demonstrate that Afadin is essential for *de novo* lumen formation in these tissues, and suggest the lumenal defects may be due to junction mislocalization.

Both Rap1 and Afadin have therefore both been shown to be crucial for correct placement of lumens and their junctions. Because Afadin is a known interactor and effector of Rap1 (11), it suggests that the role of Rap1 in lumen continuity is mediated at least in part through Afadin localization. In *Drosophila*, Rap1 and the Afadin orthologue Canoe are both essential for positioning of adherens junctions and cell polarity (19), and Rap1 regulates Afadin localization (19, 21). Our results therefore extend observations in invertebrates to mammalian epithelial tubules. Given that Rap1 and Afadin localize to adherens junctions in a wide range of organisms from flies to mammals, it is likely that these factors represent evolutionarily conserved and essential upstream regulators of cell-cell junctions and the morphogenetic processes that depend upon their interactions.

An interesting aspect of Rap1 function in the developing kidney is its differential requirement in nephron versus ureteric epithelium. We theorize that the differences in the nature of lumen formation in these tubules underlies the phenotypic differences. The MDCK cells utilized in our *in vitro* experiments are thought to be derived from cells of the distal nephron and more closely approximate nephron epithelia than ureteric epithelia (37). Prior studies have demonstrated that apical exocytosis of intracellular vesicles carrying components of the apical membrane act to initiate and expand the lumenal (apical) surface of MDCK spheroids (30). A similar mechanism has been proposed for lumen initiation in s-shaped body lumen generation in the nephron epithelia (38), which similarly undergoes polarization, apical membrane biogenesis, and nascent lumen formation. Furthermore, in the nephron epithelia, multiple tiny lumens must then interconnect to form a continuous lumen (6).

In contrast to this, the ureteric bud epithelium is already polarized and has a pre-existing lumen such that the ureteric tubule and its lumen extend by addition of new cells through cell division. Thus, even though single cell RNA-seq datasets (14) demonstrate that *Rap1a* and *Rap1b* expression (as well as *Rap2a*, *Rap2b*, and *Rap2c*) are similar in both epithelial tubule types, our study suggests that the nature of generating epithelial polarity *de novo* (as occurs in the nephron) and the connection of discrete small lumens requires Rap1. It is currently unknown which Rap1 GEFs, and GAPs are present in these cell types, and it is quite likely that differences in these proteins underlie phenotypic differences.

Although the phenotypes are similar to that of Afadin mutants, they are not identical. In addition to polarity and lumenal defects in developing nephrons, there are significant morphogenetic defects in Rap1 nephron-specific mutants. In Rap1 mutants, the s-shaped body is smaller with a thickened, lumenless section at its proximal end. These defects are qualitatively more severe than those observed in Afadin mutants. Additionally, Rap1 mutant cells have an abnormal shape, appearing more rounded than wild type nephron epithelia. Consistent with the additional phenotypes, Rap1 pups die in the neonatal stage, which does not occur with Afadin mutants. Our results are consistent with recent work demonstrating that Rap has a more profound and early effect on the morphogenesis of germband extension in the Drosophila embryo compared to loss of Afadin/Cno (39).

The underlying cause of these phenotypic differences remains unclear; however, one important observation relating to the more severe phenotype is that the membrane

localization of Rap1 *in vitro* during lumen formation is broader than that of Afadin, similar to observations in *Drosophila* epithelia (19). Taken together, these findings strongly suggest that the cell shape and morphogenetic defects of Rap1 mutants are not solely mediated by Afadin, and that additional (non-Afadin) effectors play a critical role. Indeed, Rap1 has been shown to play a role in regulating cell shape in a manner independent of Afadin (19, 40). This role may be due to known Rap1 effectors such as RIAM, which enables talin and integrin activation and is known to induce changes in cell shape (41, 42).

In summary, we have identified that Rap1 is essential for *de novo* lumen formation and tubule morphogenesis in the mammalian kidney that is partially mediated via a Rap1-Afadin axis. Future experiments to elucidate the role of Rap1 regulators such as Rap1 GEFs and GAPs and additional Rap1 effectors are likely to provide new insight into lumen formation in the kidney.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Rap1 is required for lumen continuity *in vitro* and in mouse nephron tubules.
- Rap1 also mediates normal morphogenesis of nephron tubules.
- Rap1 is dispensable for lumen continuity and morphogenesis in ureteric tubules.
- Rap1 correctly localizes its effector, Afadin, in kidney tubules.
- Rap1 functions in nephron tubules via both Afadin and additional Rap1 effectors.

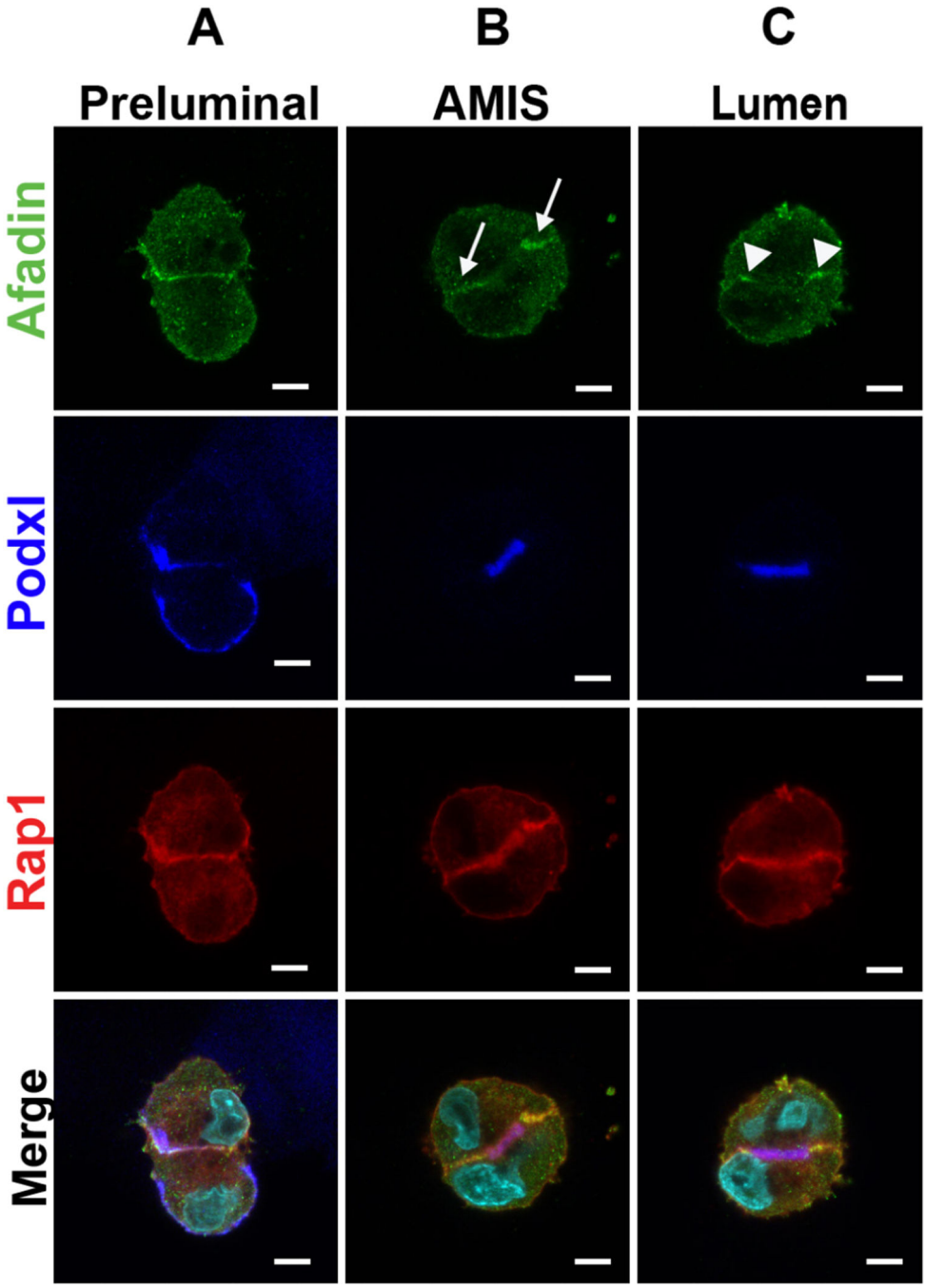

Figure 1. mCherry-Rap1a and Afadin localization during stages of lumen formation

A. Immunofluorescence of two-cell stage MDCK spheroids with Afadin (green), mCherry-Rap1a (red), and podxl (blue) prior to lumen formation.

B. Immunofluorescence as in A at the apical membrane initiation site (AMIS) stage.

C. Immunofluorescence at the lumen stage.

Arrows depict Afadin at lateral membranes and arrowheads show Afadin at apical-lateral junctions. Results are representative of at least 2 independent experiments. Scale bars: 5 μm.

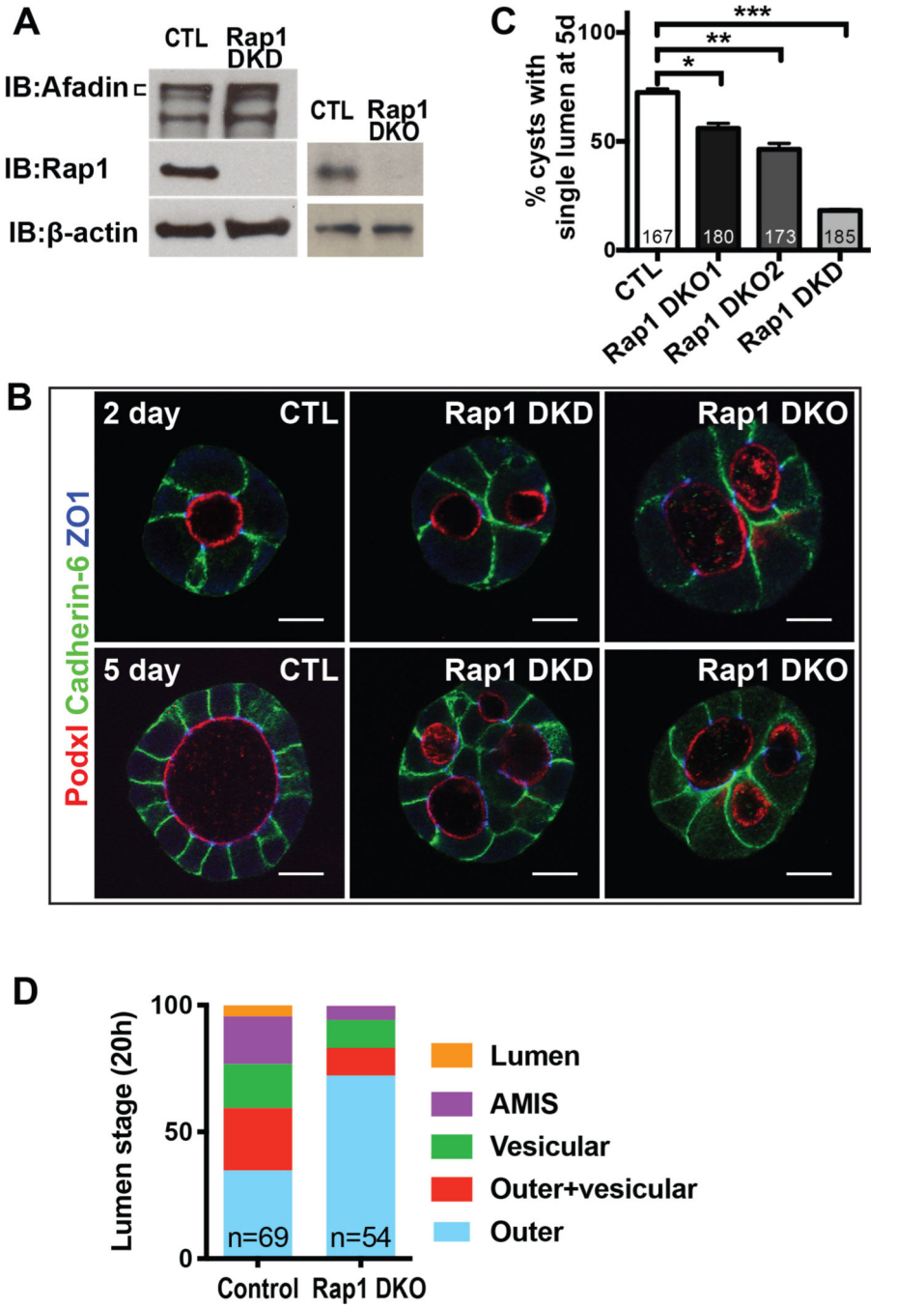

Figure 2. Rap1a/b is required for timely and normal lumen formation in spheroids

A. Western blots of Rap1a/b and Afadin in Rap1a/b double knockdown (Rap1 DKD) and double knockout (Rap1 DKO) cells.

B. Immunofluorescence of Podxl1, cadherin-6, and ZO1 in 2-day and 5-day spheroids with Rap1 DKD and DKO. The Rap1 DKD/DKO spheroids have multiple lumens. Scale bars: 10 μm.

C. Quantification of percentage of spheroids with a single lumen at 5 days. Rap1a/b DKD and DKO spheroids have a lower percentage with a single lumen. Results from two double DKO clonal lines are shown.

D. Quantification of the percentage of two cell spheroids at the indicated lumen stages at 20h. See Fig 1A and text for details. Rap1a/b DKO spheroids have fewer in the AMIS and lumen stages, indicating delay in lumen formation.

Results are representative of at least 2 independent experiments.

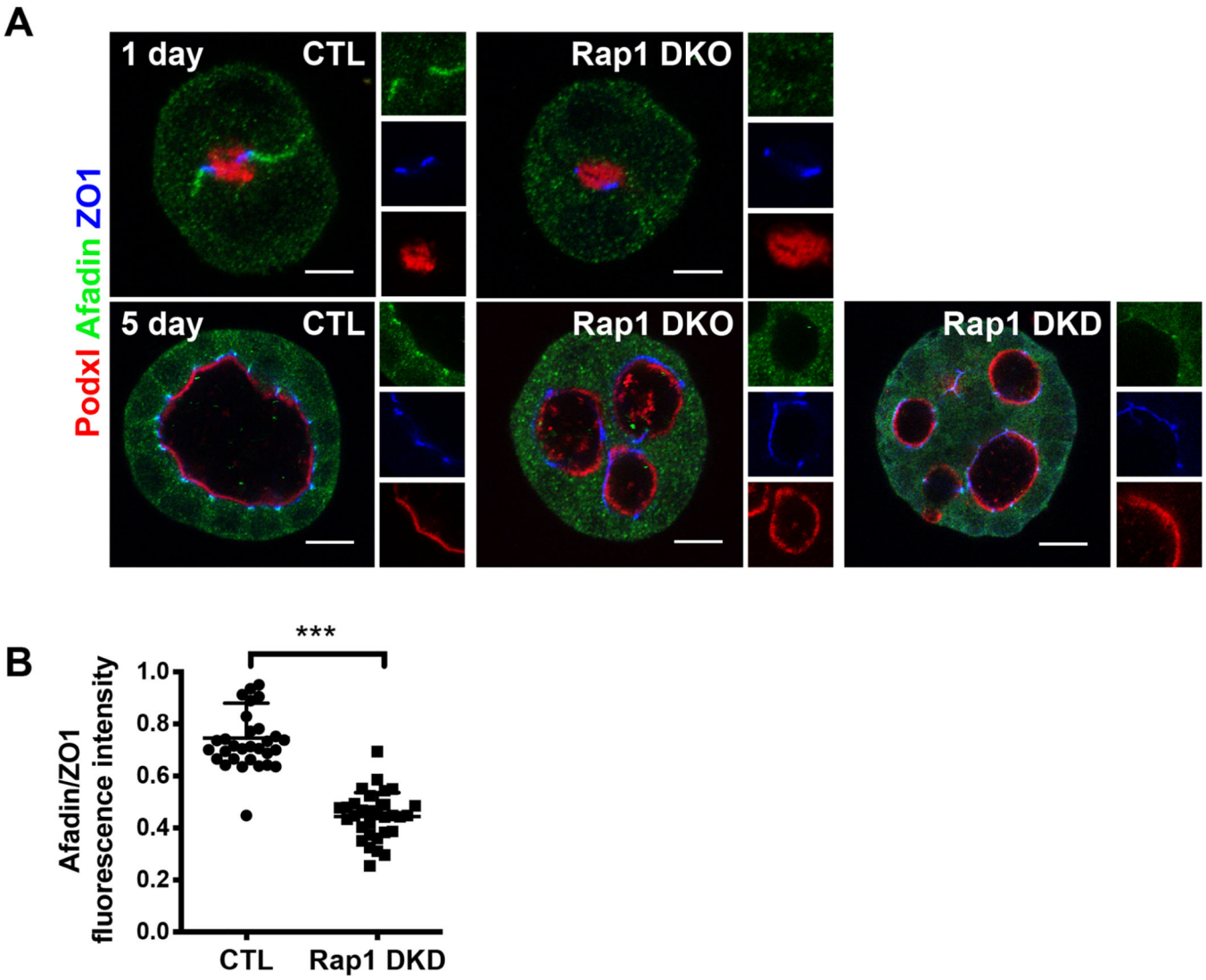

Figure 3. Rap1a/b is required for recruitment of Afadin to cellular junctions in spheroids.

A. Immunofluorescence with Podxl1, Afadin, and ZO1 in 1-day (20 hours) and 5-day spheroids. Rap1a/b DKD and DKO spheroids had loss of Afadin localization to the lateral cell junctions at day 1 and apical-lateral junctions at day 5. Scale bars: 5 μm (upper panels), 10 μm (lower panels).

B. Quantification of the Afadin/ZO1 ratios at apical-lateral junctions by fluorescence intensity (***p<0.001, 95% CI −0.36 to −0.24).

Results are representative of at least 2 independent experiments.

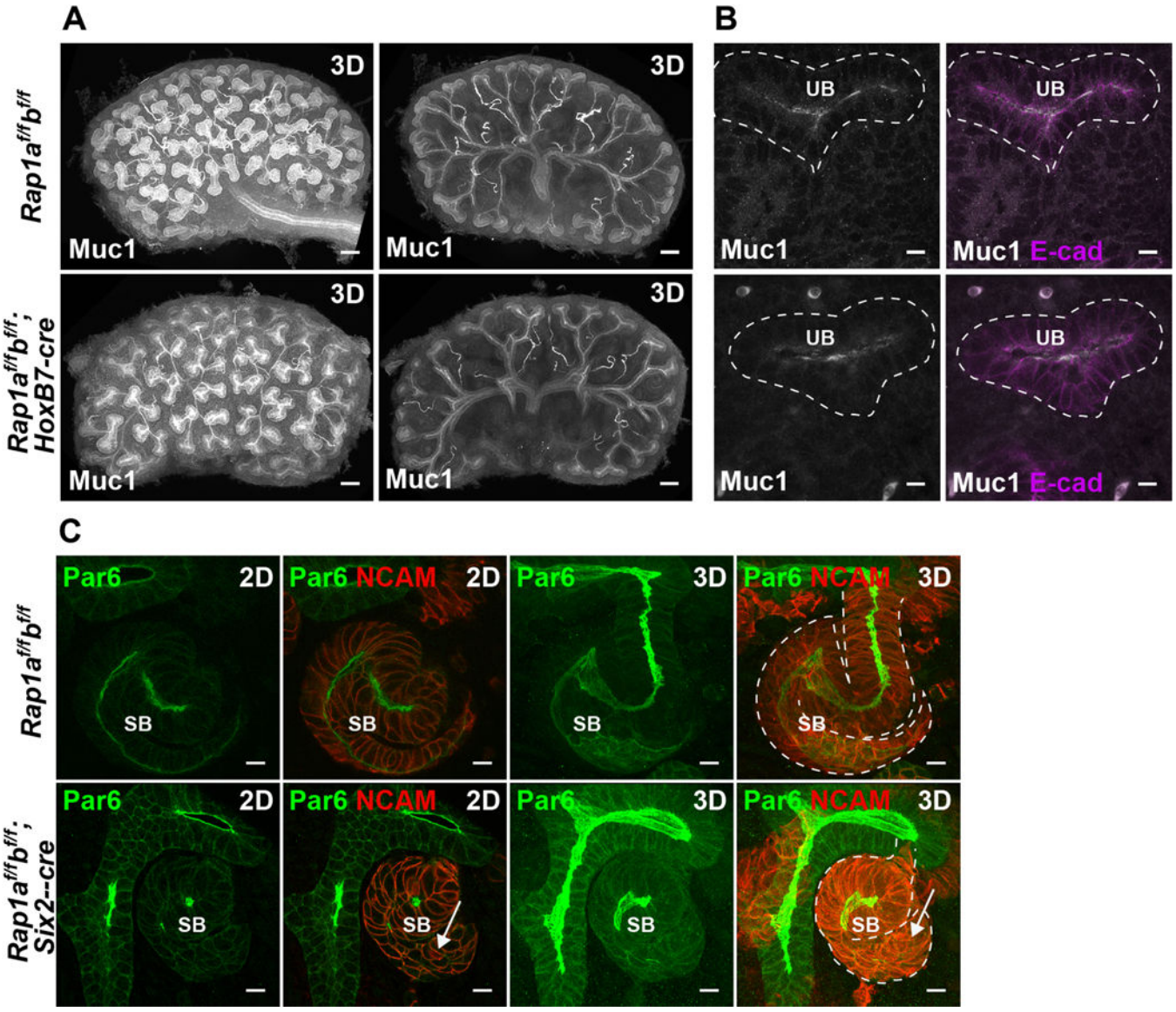

Figure 4. Absence of Rap1 causes lumenal and morphogenetic defects in nascent lumen formation of developing nephron tubules but not ureteric-bud tubules

A. E14.5 control (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$) and mutant (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; Hoxb7-cre*) kidneys were immunostained with Muc1 (white) which labels ureteric bud lumens and distal tubule lumens. Left images are 3D projections from a confocal z-stack (z is 390 μm). Right images are 3D projections from the central images (130 μm), which allows better visualization of the ureteric bud and stalk lumens. Scale: 100 μm.

B. High magnification images of immunofluorescent staining with Muc 1 (white) and E-cadherin (magenta) depicting the ureteric bud (UB) epithelia and lumens. Scale: 10 μm.

C. E14.5 control (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$) and mutant kidneys lacking Rap1a/b from metanephric mesenchyme and its derivatives (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; Six2-cre*) were immunostained with Par6 (green) and NCAM (red). Par6 marks the lumens and NCAM delineates the metanephric mesenchyme and s-shaped bodies (SB). Single images and their projected

z-stacks (6 μm) are indicated as 2D and 3D, respectively. Note the abnormal shape of the s-shaped body and lack of a continuous lumen in the mutant (arrows). Scale: 10 μm.

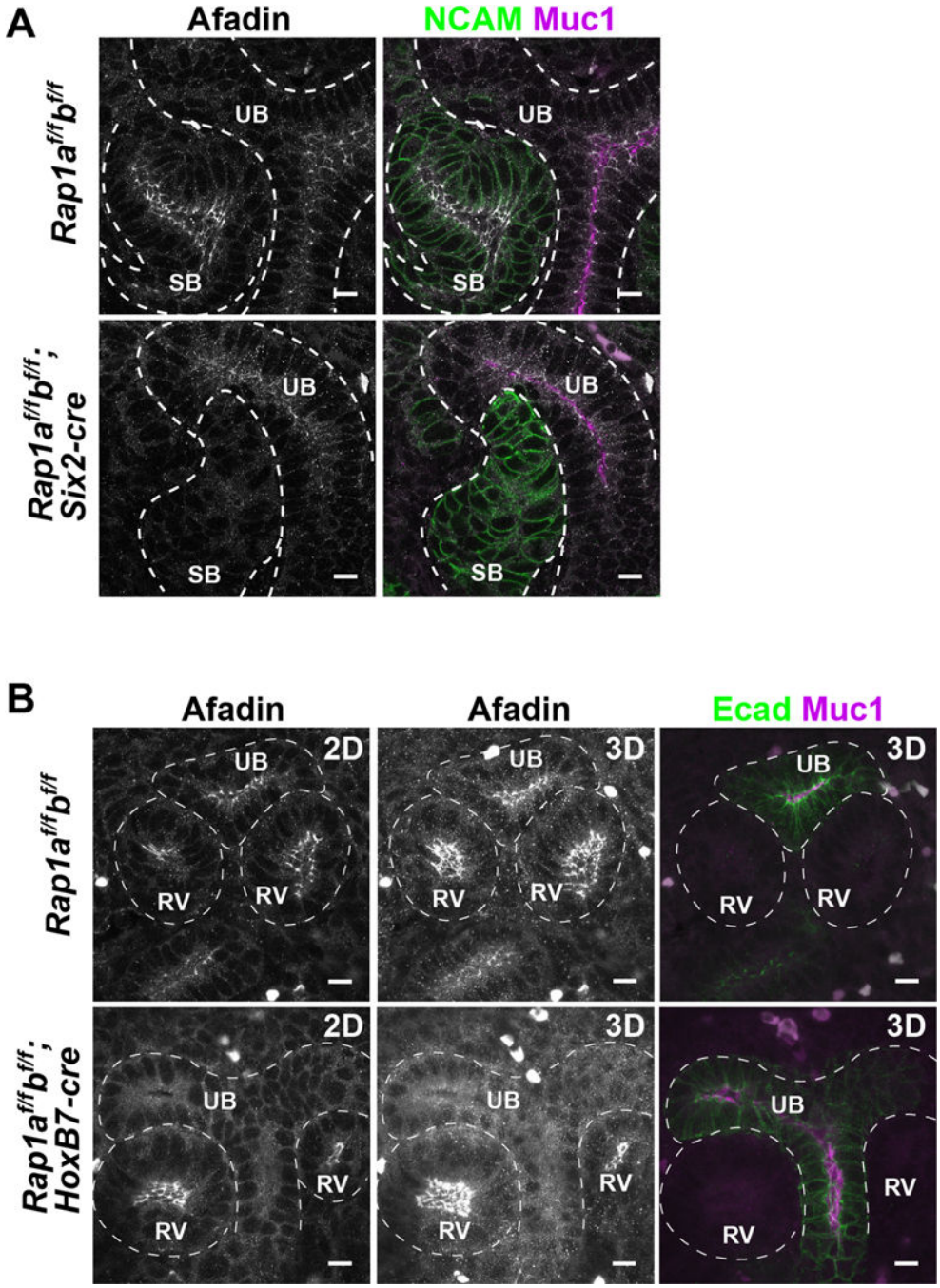

Figure 5. Absence of Rap1 leads to mislocalization of Afadin in vivo in nephron and ureteric bud epithelia.

A. E14.5 control (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$) and mutant (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; Six2-cre*) kidneys lacking Rap1a/b from metanephric mesenchyme were immunostained with Afadin (white), NCAM (green) and Muc1 (magenta). Scale: 10 μm.

B. E14.5 control (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$) and mutant (*Rap1a*$^{f/f}$*; Rap1b*$^{f/f}$*; HoxB7-cre*) kidneys lacking Rap1a/b from metanephric mesenchyme were immunostained with Afadin (white), E-cadherin (green) and Muc1 (magenta). Single images and projected z-stacks (6 μm) are indicated as 2D and 3D, respectively. Scale: 10 μm.

Results are representative of n = 3 for each genotype.