Inversin modulates the cortical actin network during mitosis

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Am J Physiol Cell Physiol 305: C36–C47, 2013. First published March 20, 2013; doi:10.1152/ajpcell.00279.2012.—Mutations in inversin cause nephronophthisis type II, an autosomal recessive form of polycystic kidney disease associated with situs inversus, dilatation, and kidney cyst formation. Since cyst formation may represent a planar polarity defect, we investigated whether inversin plays a role in cell division. In developing nephrons from inv−/− mouse embryos we observed heterogeneity of nuclear size, increased cell membrane perimeters, cells with double cilia, and increased frequency of binuclear cells. Depletion of inversin by siRNA in cultured mammalian cells leads to an increase in bi- or multinucleated cells. While spindle assembly, contractile ring formation, or furrow ingression appears normal in the absence of inversin, mitotic cell rounding and the underlying rearrangement of the cortical actin cytoskeleton are perturbed. We find that inversin loss causes extensive filopodia formation in both interphase and mitotic cells. These cells also fail to round up in metaphase. The resultant spindle positioning defects lead to asymmetric division plane formation and cell division. In a cell motility assay, fibroblasts isolated from inv−/− mouse embryos migrate at half the speed of wild-type fibroblasts. Together these data suggest that inversin is a regulator of cortical actin required for cell rounding and spindle positioning during mitosis. Furthermore, cell division defects resulting from improper spindle position and perturbed actin organization contribute to altered nephron morphogenesis in the absence of inversin.


Am J Physiol Cell Physiol 305: C36–C47, 2013. First published March 20, 2013; doi:10.1152/ajpcell.00279.2012. — Mutations in inversin cause nephronophthisis type II, an autosomal recessive form of polycystic kidney disease associated with situs inversus, dilatation, and kidney cyst formation. Since cyst formation may represent a planar polarity defect, we investigated whether inversin plays a role in cell division. In developing nephrons from inv−/− mouse embryos we observed heterogeneity of nuclear size, increased cell membrane perimeters, cells with double cilia, and increased frequency of binuclear cells. Depletion of inversin by siRNA in cultured mammalian cells leads to an increase in bi- or multinucleated cells. While spindle assembly, contractile ring formation, or furrow ingression appears normal in the absence of inversin, mitotic cell rounding and the underlying rearrangement of the cortical actin cytoskeleton are perturbed. We find that inversin loss causes extensive filopodia formation in both interphase and mitotic cells. These cells also fail to round up in metaphase. The resultant spindle positioning defects lead to asymmetric division plane formation and cell division. In a cell motility assay, fibroblasts isolated from inv−/− mouse embryos migrate at half the speed of wild-type fibroblasts. Together these data suggest that inversin is a regulator of cortical actin required for cell rounding and spindle positioning during mitosis. Furthermore, cell division defects resulting from improper spindle position and perturbed actin organization contribute to altered nephron morphogenesis in the absence of inversin.

INHERITED POLYCYSTIC KIDNEY DISEASE (PKD) is a heterogeneous group of genetic diseases characterized by defects in the organization of the tubular epithelium that lead to expansion of the tubular lumen and cyst formation (4, 37). Nephronophthisis is an autosomal recessive form of chronic kidney disease that represents the leading genetic cause for kidney defects in children and young adults. Inversin (INVS) mutations have been linked to an infantile form of nephronophthisis (nephronophthisis type II), and loss of functional inversin in mice and zebrafish leads to the formation of renal cysts (10, 22, 28). Like most renal cyst-associated proteins, inversin has been shown to localize to primary cilia (19, 26, 28). The commonality of cilia localization for cyst-associated proteins supports the view that the common pathology of genetic defects in genes involved in different forms of PKD can be explained by their role in cilia formation or cilium-associated signaling (12, 40). While inversin does not appear to be required for cilia organization (30), a potential role for inversin during Wnt signaling has been reported (36).

The Wnt signaling pathway is an essential developmental pathway that branches into canonical and noncanonical signaling mechanisms. During canonical Wnt signaling, Wnt binding is sensed by cell surface receptors of the Frizzled family which leads to the activation of Disheveled (dsh in Drosophila, dvl in the mouse). This interaction prevents the degradation of β-catenin and induces β-catenin-dependent transcription of downstream TCF/LEF target genes (36). In contrast, during noncanonical Wnt signaling, Dvl signals to downstream effectors from the Rho GTPase family that induces the rearrangement of the actin cytoskeleton (9, 32). This later pathway is required for establishment of proper planar cell polarity (PCP). Remarkably, transgenic overexpression of β-catenin in mice leads to the formation of kidney cysts, supporting a potential role for the Wnt signaling cascade during kidney development and renal cyst formation (31).

Inversin is a member of the large family of ankyrin repeat-containing proteins, with the human isoform harboring 16 tandem ankyrin repeats as well as two D-Box destruction domains and two calmodulin binding IQ domains. Inversin binds to APC2, a component of the APC complex (APC/C) and Dvl. Inversin binding to APC2 is dependent on the first D-Box sequence, leading to APC/C-dependent degradation of Dvl. Furthermore, a patient with a mutation in this D-Box failed to properly degrade Disheveled. These observations led to the proposal that inversin is involved in the regulatory balance of canonical and noncanonical Wnt signaling by targeting a cytoplasmic pool of Disheveled for APC/C-dependent degradation (19, 36). This would place inversin at a central junction of canonical and noncanonical Wnt signaling, suggesting a potential role for inversin in regulating the balance between these two signaling cascades. However, the downstream mechanisms and potential targets of inversin-dependent Wnt signaling are largely unidentified.

We have previously reported that in addition to its association with primary cilia, inversin also localizes to the cell cortex in cultured cell monolayers and forms a complex with N-cadherin and the catenins, suggesting a role for inversin in the formation of cell adhesions (23, 34). Furthermore, inversin also localizes to centrosomes and spindle microtubules in mitotic cells and inversin can bind microtubules in vitro, thus indicating a potential role for inversin during mitosis (19, 26). To test whether inversin is involved in the regulation of mitotic processes, we investigated the role of inversin during mitotic events such as mitotic spindle assembly, contractile ring formation, rearrangement of the actin cytoskeleton and cell division. We find that loss of inversin leads to an increase of...
multinucleated cells. While contractile ring assembly and spindle organization or microtubule dynamics appear largely unperturbed, loss of inversin leads to a drastic reorganization of the cortical actin cytoskeleton that prevents cell rounding during mitosis and leads to spindle positioning defects.1

MATERIALS AND METHODS

Animal care and housing. All animals were housed in the vivarium at Indiana University School of Medicine, and all experiments were performed with the approval of the Indiana University Animal Care and Use Committee.

Plasmids and RNAi. Plasmids for cRhoA green fluorescent protein (GFP) chimera, amidin-GFP, tubulin-GFP, and mLC-GFP were generously provided by A. Pieky (Concordia University, Montreal, CA). Plasmid containing EB-1 GFP and CLIP-170 GFP were a gift from H. Goodson (University of Notre Dame, South Bend, IN). Plasmid bearing eGFP-inversin chimeric protein was supplied by P. Overbeek ( Baylor College of Medicine, Houston, TX). JN/S siRNA was designed as suggested by the manufacturer (SASI_Hs01_00111914, Sigma-Aldrich, St. Louis, MO) (targets covered NM_014425.2, NM_183245.1, sense strand GCACUAGCGUCAGAGAUUUdTdT, anti-sense strand ACUCUGAGCAGCAUAGUGCdTdT) and examined for possible cross-reactivity using NCBI-BLAST search.

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Live cell imaging. Control or siRNA-treated cells were imaged between 40 h and 72 h posttransfection. Cells were kept at 37°C using a Warner DH-35 dish heater (Warner Instruments, Hamden, CT) and were maintained in DMEM media supplemented with 10 mM HEPES, pH 7.4. The CO2 concentration was ambient atmosphere. Images were acquired using a spinning disk confocal microscope equipped with an Andor iXon air cooled EMCCD camera (South Windsor, CT). Images were acquired every 10 s when imaging mitotic cells. Imaging of EB-1 GFP or CLIP-GFP in interphase cells was carried out by acquiring images every second for high temporal resolution of microtubule dynamics.

Two-photon confocal microscopy of embryonic kidneys. Kidney sections were blocked with 3% bovine calf serum in PBS for 1 h, followed by a 2-h incubation with a α- and β-tubulin antibody mixture in Tris-buffered saline with 0.5% Triton X-100. Hoechst 33342 was used to label DNA and rhodamine-phalloidin was used to label actin (Invitrogen). Images were acquired with either a Zeiss LSM-510 Meta Confocal/Multiphoton Microscope (Zeiss USA) or an Olympus FV1000-MPE Confocal/Multiphoton Microscope.

Image processing and quantification. Image processing and quantification were carried out using ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD). For each figure, 16-bit images were scaled the same way and transformed into 8-bit images. For all quantification experiments, control or RNAi-treated cells were fixed and stained with rhodamine-phalloidin and α/β-tubulin. For total cell area measurements, the cell membrane of metaphase cells was outlined using an ImageJ freehand tool and the area was then quantified using ImageJ. To assess the extent of spindle elongation, the distance between the two spindle poles was measured. To determine spindle positioning during metaphase, the cell centroid was determined using ImageJ, and the distance between the centroid and the spindle midpoint (center of the axis connecting the two spindle poles) was measured. For spindle positioning during anaphase, the distance between spindle midpoint and cell center (center of the cell along the spindle axis) was measured.

Specimen preparation for electron microscopy. The specimens were fixed in 2% glutaraldehyde (Polysciences, Warrington, PA) in 0.1 M phosphate buffer. After fixation the specimens were rinsed with PBS followed by postfixation with 1% osmium tetroxide in phosphate buffer for 1 h. After rinsing again with PBS the tissue specimens were dehydrated through a series of graded ethyl alcohols from 70 to 100%. After dehydration, specimens were infiltrated with two changes of 100% propylene oxide (PO) and a 50:50 mixture of PO and the embedding resin (Embed 812, Electron Microscopy Sciences, Hatfield, PA) overnight. Specimens were transferred to fresh 100%
embossing media under vacuum for 6–8 h then embedded in a fresh change of 100% embedding media. Following polymerization overnight at 60°C, blocks were sectioned. Thin sections were cut (70–80 nm), stained with uranyl acetate, and viewed on a Tecnai BioTwin (FEI, Hillsboro, OR), and digital images were taken with an AMT (Advanced Microscope Techniques, Danvers, MA) CCD camera. For scanning electron microscopy (SEM) studies, tissue segments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, postfixed in osmium tetroxide, dehydrated in a graded series of ethanol, critical point dried using a Tousimis Samdi 790 critical point dryer (Rockville MD), mounted on stubs, and sputter coated with Au/Pd using a Polaron Sputter Coater (Energy Beam Sciences, East Grampny, CT) before viewing on a JEOL 6390 SEM (JEOL USA, Peabody, MA).

Cell motility assay. Dermal fibroblasts were grown to confluence in growth media after being plated on glass bottom dishes (MatTek, Ashland, MA). On the day of the experiment the media were changed to MEM with 0.25 g/l NaHCO3, 10 mM Na HEPES, pH 7.4 and 2% bovine serum albumin and 2% fetal bovine serum. The cells were scraped with a sterile pipette tip and imaged with a Nikon TE200 inverted microscope equipped for wide field phase contrast microscopy. To identify phenotypes associated with loss of inversin, we used two-photon confocal microscopy to determine the cellular morphology of interphase and mitotic cells in the kidneys from \( \text{inv}^{-/-} \) mice. As described previously, day 1 kidneys from \( \text{inv}^{-/-} \) embryos show highly disorganized embryonic tubules with increased tubular diameter (30). Interestingly, we observed a significant increase in binuclear tubular epithelial cells (Fig. 1, A and D). Quantification of the number of binucleated tubular epithelial cells revealed a 10-fold increase over tubular cells from control kidneys (Fig. 1B). In addition, tubular epithelial cells in \( \text{inv}^{-/-} \) kidneys had increased cell area as compared with wild-type controls (Fig. 1, C and D). The assessment of the number of multinucleated cells and cell area probably underestimated the extent of cellular changes because some tubular regions were severely disorganized in \( \text{inv}^{-/-} \) kidneys. These regions were omitted from the quantification because confident identification of binucleated cells was difficult particularly since a significant number of cells appeared apoptotic. This observation was corroborated by ultrastructural analysis, revealing multinucleated cells as a readily observed phenotype in these kidneys (Fig. 2A) and cells with micronuclei (Fig. 2B).

We additionally observed that tubular cells from \( \text{inv}^{-/-} \) kidneys showed increased total cell area. Cell area measurements confirmed this observation showing a twofold increase in total cell area in tubular cells from \( \text{inv}^{-/-} \) mice over control wild-type cells (Fig. 1, C and D). Furthermore, we observed that cell-cell contacts appeared abnormal in \( \text{inv}^{-/-} \) kidneys. Instead of tight uniform contacts observed in wild-type cells we often observed that cell-cell contacts showed a ruffled pattern, suggesting that loss of inversin affected cell-cell contacts. Finally, we also observed that metaphase spindles of tubular cells were frequently displaced relative to the cell centroid in tubular cells from \( \text{inv}^{-/-} \) embryos similar to the spindle positioning defect observed in cultured mammalian cells treated with inversin siRNA (Fig. 1E). To further evaluate inversin function, we characterized the cellular phenotype associated with inversin knockdown using RNAi in cell culture.

Knockdown of inversin by RNAi leads to an increase in multinucleated cells. The proliferative defects associated with perturbed inversin function that lead to the formation of large kidney cysts, combined with the observation that inversin localizes to the mitotic spindle, suggested a potential role for inversin during mitosis. To investigate this possibility, we initially decided to knock down endogenous inversin in cultured cells to determine whether loss of inversin leads to mitotic defects. As evidenced by immune blots, we were able to reduce endogenous inversin levels by >90% in both HeLa and HEK-293 cells (Fig. 3A). We then quantified mitotic defects by counting the proportion of bi- or multinucleated cells in fixed samples after 48 h of RNAi treatment. We observe a 2.5-fold increase in multinucleated cells in cells treated with INV5 RNAi as compared with control RNAi-treated cells (Fig. 3B, \( P < 0.03 \)). Similarly, we found that 14% of mouse dermal fibroblasts isolated from \( \text{inv}^{-/-} \) pups were multinucleated compared with 3% of wild-type dermal fibroblast (\( P < 0.002 \)). Rescue experiments with full-length GFP-tagged inversin in embryonic fibroblast from \( \text{inv}^{-/-} \) mice decreased the percentage of multinucleated cells to 6.5% close to wild-type levels and decreased the percentage of multinucleated cells in GFP-rescued \( \text{inv}^{-/-} \) cells (Fig. 3C, \( P < 0.05 \)). Taken together, these data show that inversin loss leads to an increase in mitotic defect frequency.

Inversin is required for proper cell rounding during mitosis by regulating the organization of cortical actin. Prior studies have reported that inversin is recruited to the mitotic spindle (27). We confirmed this observation in HeLa cells by staining HeLa cells during mitosis with anti-inversin antibodies. In Fig. 4, inversin localized to the spindle poles and was also located in diffuse cytoplasmic puncta in a M phase HeLa cell. This finding is in agreement with prior published results (27). To determine the nature of the mitotic defect that led to an increase in multinucleated cells, we followed inversin-depleted mitotic cells using live cell microscopy. In contrast to control cells that acquired a spherical shape during metaphase, inversin-depleted metaphase cells remained partially flattened, exhibited significantly larger cell areas than control cells, and in some cases had drastically asymmetric shape. Although RNAi-treated cells partially rounded up upon entering anaphase, they still exhibited increased cell surface areas (Fig. 5A). We quantified the extent of the cell rounding defect by measuring total cell area of fixed inversin-depleted cells in metaphase and comparing it to control cells. Inversin-depleted cells show a 2.5-fold increase in total cell area illustrating defects in cell rounding (Fig. 5B, \( P < 0.001 \)). Despite this cell rounding defect the cytokinetic furrow ingresses normally midway between the spindle poles. Interestingly, mitotic spindles were often misplace leading to the formation of two nascent daughter cells of unequal size.

Cortical actin dynamics and distribution of focal adhesions are perturbed in the absence of inversin. To test whether the change in cell shape and the failure of inversin-depleted cells to round up properly were a consequence of failure to reorga-
Fig. 1. Embryonic kidneys from inv<sup>−/−</sup> mice had increased numbers of multinucleated tubular cells with larger cell area and misplaced metaphase spindles. 

A: two-photon microscope image of a P1 embryonic kidney from inv<sup>−/−</sup> mouse. Arrow indicates multinucleated cell. All two-photon images in this figure are from kidneys fixed with 3% paraformaldehyde pH shift method and stained with rhodamine-phalloidin and Hoescht 33342. Bar, 10 μm. 

B: quantification of the amount of multinucleated cells in tubules from inversin-knockout kidneys compared with control kidneys. 

C: two-photon microscope images of tubular cells from control and inv<sup>−/−</sup> kidneys. Note the increase in cell area in the absence of inversin. Punctate staining in the middle of the control cells is due to nucleoli staining by Hoechst 33342. 

D: quantification of average cell area of tubular kidney cells of control or inv<sup>−/−</sup> kidneys. WT, wild type. 

E: representative images from control (left) and inv<sup>−/−</sup> kidneys (right) stained with rhodamine-phalloidin, Hoescht 33342, and αβ-tubulin antibodies. Note that there are spindle positioning alterations relative to the cell centroid in metaphase cells in kidneys from inv<sup>−/−</sup> mice (arrows). Insets: higher magnification of mitotic cells shown below the control (left) and inv<sup>−/−</sup> kidney (right).
nize the actin cytoskeleton, we used phalloidin labeling to visualize the organization of the actin cytoskeleton. In contrast to control cells, where actin accumulated uniformly around the cellular cortex during metaphase before becoming enriched in the equatorial plane and in the ingressing cleavage furrow, we observed large filopodia-like structures, reminiscent of retraction fibers, surrounding the entire cell cortex. These actin fibers persisted in cortical regions of anaphase cells and rapidly spread around the entire cortex during telophase when cells flattened out (Fig. 6A). Remarkably, we also observed a similar increase of cortical filopodia like structures in all interphase cells suggesting that the effect of inversin loss on the organization of the cortical actin cytoskeleton is not restricted to mitosis (Fig. 6A).

We used antibodies against a filopodia component, paxillin, to determine whether the observed cortical actin fibers were filopodia (6, 17). Indeed, paxillin localized to these cortical actin-rich extensions in both interphase and mitotic cells (Fig. 6B). Furthermore, we also observed localization of α-actinin to the base of these filopodia (data not shown), confirming the identity of those actin extensions. Remarkably, we also observed a significant redistribution of focal adhesions from the cell body to the cell periphery in inversin-depleted cells (Fig. 6B), suggesting that inversin may be involved in regulating cellular adhesion. To test whether this translates into defects in cell motility, we performed scratch-wound assays on wild-type and mutant fibroblasts. Indeed, inv−/− fibroblasts showed significantly reduced motility (17.1 ± 8.3 nm/min) than control fibroblasts (5.4 ± 4.2 nm/min; P < 0.005).

Loss of inversin does not affect contractile ring assembly, furrow ingestion, or contractile organization. To determine whether inversin plays a role in other mitotic processes, we examined whether loss of inversin results in defects in contractile ring formation or spindle assembly. We found that loss of inversin did not affect contractile ring assembly or furrow ingestion since contractile ring proteins RhoA, myosin light chain, and anillin all localized normally to the ingressing furrow (Fig. 7A). Since inversin binds to microtubules in vitro and to the mitotic spindle in cultured mammalian cells (19, 26, 28), we hypothesized that inversin regulates spindle assembly or microtubule dynamics during mitosis. However, inversin-depleted cells formed proper bipolar spindles, exhibited wild-type levels of spindle elongation, and had no apparent defects in microtubule dynamics as determined by measurements of mitotic spindle length at metaphase (Fig. 7B). We extended these data by examining CLIP-170 and EB-1 movement along microtubules and observed no obvious defect in movement of either protein along microtubule tracks in inversin-depleted cells (Supplemental Fig. S1; Supplemental Material for this article can be found online at the Journal website).

Inversin is required for proper spindle positioning during mitosis. Since we observed unequal cell divisions in inversin-depleted cells, we quantified spindle positioning during metaphase by measuring the distance between the cell centroid and the spindle midpoint, which we define as the midpoint between the two spindle poles (20, 27, 28). In contrast to control cells where mitotic spindles were in close proximity of the cell centroid, the majority of mitotic spindles were significantly displaced in inversin-depleted cells. These positioning defects are variable and can be quite dramatic as illustrated in Fig. 8A. Plotting the distance of the spindle midpoint relative to the cell centroid as a function of total cell area revealed a partial correlation of the spindle positioning defect with the extent of the defect in cell rounding, suggesting that spindle misalignment is a consequence of defective cell rounding (Fig. 8B). Furthermore, measuring the distance between the spindle mid-point and cell center along the spindle axis revealed marked asymmetry in positioning of the spindle as compared with control cells (Fig. 8C). This asymmetric position of the mitotic spindle results in the unequal division of the cell into two daughter cells of slightly different cell area.

DISCUSSION

Inversin is the product of the Invs gene first identified by a random insertion mutagenesis screen (18, 22, 43). It localizes
to adherens junctions and the ciliary axoneme in epithelial cells and acts as a master switch for Wnt signaling by targeting Dsh for degradation (24, 27, 34, 36). The underlying mechanism(s) of kidney cyst formation as a consequence of inversin loss is still unidentified (38). In this study we show that inversin is a critical regulator of the cortical actin cytoskeleton required for proper cell rounding and spindle positioning during mitosis as well as maintenance of the tubular epithelia integrity.

In analyzing the cellular response to inversin knockdown we determined that inversin is a critical regulator of the cortical actin network. Notably, we observed formation of cortical filopodia in both mitotic and interphase cells when inversin was depleted.

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**Fig. 3.** Loss of inversin leads to an increase in bi- and multinucleated cells. A: immune blot of extracts from control cells and cells treated with INVS siRNA for 48 h, probed with inversin antibody as well as β-tubulin (loading control). B: amount of bi- or multinucleated cells determined by immunofluorescence in control (scrambled RNAi) vs. INVS RNAi-treated cells. Averages of at least three independent experiments scoring at least 500 cells per experiment are shown. Error bars represent standard deviation in all figures. Difference in percentage of multinucleated cells in wild-type versus inv−/− cells was statistically significant (P = 0.00187). C: relative amount of bi- or multinucleated cells in wild-type dermal fibroblast, dermal fibroblasts from inv−/− embryos (untransfected group), inv−/− dermal fibroblast transfected with a green fluorescent protein (GFP)-only control plasmid, or transfected with GFP-tagged full-length inversin as determined by immunofluorescence. Note that dermal fibroblast exhibited a significant increase in bi- or multinucleated cells when compared with wild-type dermal fibroblast (P = 0.00187). Rescue experiments with GFP-tagged full-length inversin in inv−/− embryonic fibroblasts led to a significant reduction of bi- or multinucleated cells when compared with untransfected inv−/− cells (P = 0.0061) or inv−/− cells transfected with a GFP control plasmid (P = 0.0495).

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**Fig. 4.** Immunofluorescence localization of inversin in a mitotic HeLa cell. Left: inversin staining showing localization at the spindle poles and in cytoplasmic puncta. Middle: Hoescht 33342 staining of metaphase chromosomes. Right: merged image from left and middle images. Inversin staining is shown in the red channel with Hoescht 33342 staining shown in the blue channel.
These phenotypes were accompanied by defects in cell rounding upon entry into mitosis. Mitotic cell rounding is an important step during cell division and has been shown to be dependent on actin-binding proteins such as moesin and is accompanied by cortical actin cytoskeleton remodeling (15, 39). In addition, there is a failure to suppress filopodia formation during entry into mitosis, a phenotype associated with a failure of paxillin degradation during entry into M phase (41, 42). Remarkably, focal adhesion proteins such as paxillin or \( \alpha \)-actinin were partially redistributed from the cell body to filopodia at the cellular cortex in inversin-depleted cells, suggesting that inversin is involved in cell adhesion to the substratum. Consistent with these observations, \( inv^{-/} \) fibroblasts showed significantly reduced motility when compared with control fibroblasts.

The observed defects in mitotic cell rounding were accompanied by significant spindle positioning defects. Spindle asymmetry correlated with the cell rounding defect, suggesting that the positioning defect is related to a failure to correctly position aster microtubule contacts. This may be due to a failure to reorganize the cortical actin cytoskeleton. Similar positioning defects have been reported in the absence of other actin regulatory proteins such as cofilin or moesin (1, 5, 13, 16). However, we did not observe defects in mitotic spindle assembly, nor did we detect drastic changes in microtubule dynamics. It is possible that our assay sensitivity for microtubule dynamics, i.e., using CLIP-170 or EB-1 to examine microtubule ends, failed to reveal subtle changes in microtubule stability (Supplemental Fig. S1). Given the cross talk between microtubules, actin cytoskeleton, and the interaction of inversin with microtubules, further experiments may be needed to exclude a subtle role for inversin in regulating microtubule dynamics (20).

The cause of the defect leading to multinucleated or binucleated cells is not entirely clear. It is likely that cytokinetic defects are a consequence of cell rounding and/or spindle positioning.
positioning defects since cytokinetic defects have been reported when either of these processes was compromised (11, 16). We did not observe failure of contractile ring assembly as evidenced by normal localization of key contractile ring proteins accompanied by normal furrow ingression. One possible cause for the multinuclear phenotype is that cell abscission is compromised as a consequence of asymmetric spindle positioning and subsequent unequal cell division. Another possi-

Fig. 6. Inversin is required for cortical actin organization during mitosis. A: representative cells from different mitotic stages stained with αβ-tubulin, rhodamine-phalloidin, and Hoechst 33342. Control RNAi-treated cells had a rounded membrane without filopodia during mitosis. Inversin-depleted cells formed extensive filopodia-like structures throughout mitosis. B: control RNAi-treated and inversin-depleted fixed cells stained with anti-paxillin antibody and rhodamine-phalloidin. The cortical actin cytoskeleton was perturbed in interphase cells, and paxillin was redistributed to the cell periphery in the absence of inversin. Note that paxillin localized to filopodia-like structures in both mitotic and interphase cells (arrows).
bility is that the increase in total cell area, or changes in cortical rigidity due to the defects in cortical actin reorganization prevent proper abscission. Given that inversin binds to the APC2 subunit, another explanation is that cell cycle progression or cell cycle timing is defective due to alterations in anaphase promoting complex activity as a consequence of inversin depletion (20). Further work is needed to evaluate this possibility.

Most notably, mitotic phenotypes similar to those observed in cell culture systems were also observed in kidney tissue derived from \( \text{inv}^{-/-} \) mice, which provided insights into the mechanisms of cyst formation. We found a marked increase in multinucleated cells using both two-photon microscopy and SEM, suggesting that cytokinetic defects contribute to cyst formation. Interestingly, SEM micrographs reveal that multinucleated cells in kidney cysts from \( \text{inv}^{-/-} \) mice frequently exhibit more than one primary cilium. This raises the interesting possibility that cyst formation in \( \text{inv}^{-/-} \) mice is the consequence of aberrant primary cilium-associated signaling, due to the presence of additional primary cilia in tubular cells that fail to divide normally.

It has been suggested that alterations in planar polarity signaling are responsible for cyst formation (7, 8). One manifestation of planar polarity alterations is randomization of
mitotic spindle orientation relative to the tubule lumen, an effect demonstrated in a PKD1 murine knockout model. Given the extent of the cystic phenotype in inv<sup>−/−</sup> mice we were unable to determine spindle orientation relative to the lumen as done by Fischer et al. (7) and Sugiyama et al. (38). Nevertheless, we observed a significant misalignment of metaphase spindles relative to the cell centroid in tubular kidney cells from inv<sup>−/−</sup> mice, suggesting that similar spindle positioning defects could cause extension of the tubular lumen and cyst formation. Additionally, Dvl-dependent regulation of PCP signaling has been frequently associated with modulation of the actin cytoskeleton (14). In this context, similar to what we observe in tissue culture, overall cell area of tubular epithelial cells was significantly increased and the lateral membrane of the epithelial cells lacked the smooth regular outline of renal epithelial cells observed in wild-type littermates. Since inversin interacts with N-cadherin at adherens junctions it is conceivable that inversin contributes to epithelial integrity by stabilizing cell-cell contacts directly through its interaction with N-cadherin or by regulating the cytoplasmic actin network through regulation of cellular levels of Dvl.

The current unifying model for PKD suggests that cyst formation is a consequence of defects in cilia-dependent processes. In this context, inversin, whose localization pattern includes prominent localization to the base of the ciliary axoneme (27, 34), has been proposed to be a master switch for canonical and noncanonical Wnt signaling by targeting Dsh for APC/C-dependent degradation by proteosomes (3, 36). Based on our findings we propose that inversin plays several roles during kidney development as a consequence of its role in regulating the actin cytoskeleton. First, inversin is responsible for actin cytoskeleton reorganization necessary for orienting spindle positioning to allow for convergent extension along the tubule axis in response to planar cell polarity signals. Second, inversin-dependent actin reorganization during mitosis is required for mitotic cell rounding and completion of cytokinesis. Third, inversin stabilizes focal adhesions and adherens junctions on the lateral and basal membrane to maintain epithelial

Fig. 8. Inversin is required for proper spindle positioning during metaphase and anaphase. A: inversin-depleted cells stained with rhodamine-phalloidin (red), α/β-tubulin (green), and Hoescht 33342 (blue) illustrating the extent of spindle positioning defects observed during metaphase. B: quantification of the spindle positioning defect by measurement of the distance between the cell centroid (orange) and the spindle midpoint (yellow). Scatterplot shows values for individual measurements as a function of cell area. Note that mitotic spindles were significantly displaced in inversin-depleted cells and that the extent of displacement correlated with cell size (P = 6.008 E-12). C: quantification of spindle positioning defects during anaphase by measurement of the distance (d) between spindle midpoint (orange) and cell center (black dot). Scatterplot shows spindle displacement from the cell center relative to total cell length (P = 3.874 E-6).
integrity. Therefore, cystogenesis due to a lack of functional inversin is caused by a compromised regulation of cellular actin dynamics. This leads to multiple defects during kidney development such as defective PCP signaling due to misaligned spindles, cell rounding and cytokinesis defects as well as loss of epithelial integrity.

Finally, the observation that inversin inhibits filopodia formation and the severity of the inv−/− phenotype in mice suggest that inversin may be required for regulation of actin dynamics outside of its role in kidney development. It will be interesting to define inversin’s role in other filopodia-dependent processes such as cell migration, wound healing, or axon guidance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES