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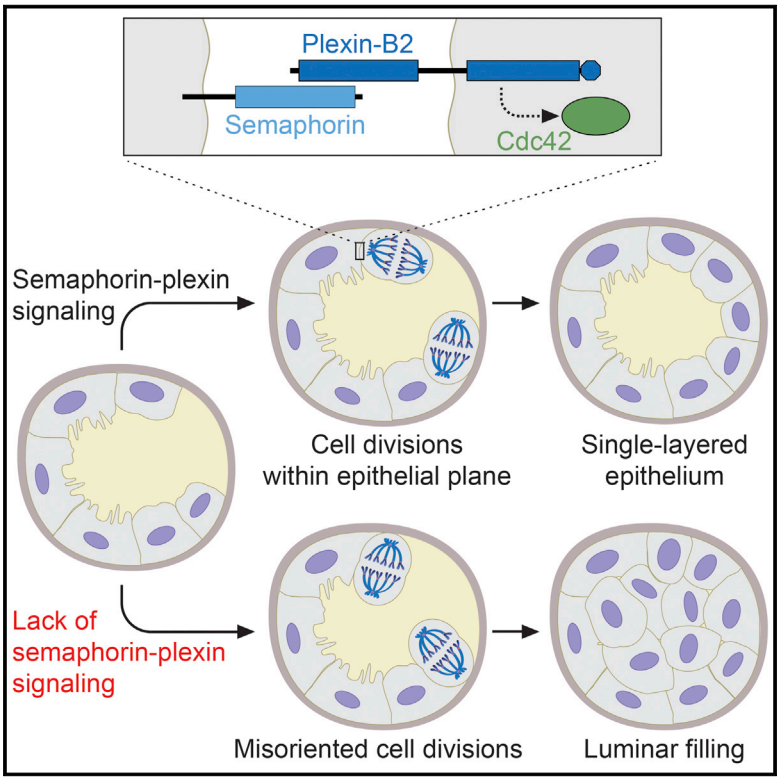
Developmental Cell

Semaphorin-Plexin Signaling Controls Mitotic Spindle Orientation during Epithelial Morphogenesis and Repair

Graphical Abstract



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In Brief

Epithelial architecture relies on the accurate orientation of cell divisions, which is determined by the mitotic spindle axis. Xia et al. show that during kidney morphogenesis and repair, proper alignment of the mitotic spindle axis with the epithelial plane requires communication among epithelial cells via the semaphorin-plexin signaling system.

Highlights

- Epithelial cell-cell communication via plexins orients the mitotic spindle
- Loss of Plexin-B1 and Plexin-B2 results in defective kidney morphogenesis
- Semaphorin-Plexin-B2 signaling is essential for kidney repair
- Plexin-B2 signals through its GAP domain and Cdc42 to orient the mitotic spindle

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Semaphorin-Plexin Signaling Controls Mitotic Spindle Orientation during Epithelial Morphogenesis and Repair

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SUMMARY

Morphogenesis, homeostasis, and regeneration of epithelial tissues rely on the accurate orientation of cell divisions, which is specified by the mitotic spindle axis. To remain in the epithelial plane, symmetrically dividing epithelial cells align their mitotic spindle axis with the plane. Here, we show that this alignment depends on epithelial cell-cell communication via semaphorin-plexin signaling. During kidney morphogenesis and repair, renal tubular epithelial cells lacking the transmembrane receptor Plexin-B2 or its semaphorin ligands fail to correctly orient the mitotic spindle, leading to severe defects in epithelial architecture and function. Analyses of a series of transgenic and knockout mice indicate that Plexin-B2 controls the cell division axis by signaling through its GTPase-activating protein (GAP) domain and Cdc42. Our data uncover semaphorin-plexin signaling as a central regulatory mechanism of mitotic spindle orientation necessary for the alignment of epithelial cell divisions with the epithelial plane.

INTRODUCTION

The correct orientation of cell division is fundamental for the development, maintenance, and repair of epithelial tissues and relies on the mitotic spindle axis (Morin and Bellaïche, 2011). In symmetrically dividing epithelial cells, the orientation of the mitotic spindle axis within the epithelial plane is under the control of extracellular cues and their respective receptors (Gillies and Cabernard, 2011). A signaling system critical for conveying such positional information is the Wnt-Frizzled planar cell polarity pathway (PCP). In mice, it has been shown to orient epithelial cell divisions in developing tubules of the kidney along their proximodistal axis to maintain tubule diameter during tubule elongation (Carroll and Yu, 2012; Castanon and González-Gáitan, 2011). In murine nephrons, cell division orientation along the same axis is also regulated by the Fat-Dachsous (Ds) pathway to control tubule diameter (Saburi et al., 2008).

Importantly, the establishment and maintenance of epithelial architecture requires symmetrically dividing epithelial cells to not only orient within the epithelial plane but also align their division axis with the epithelial plane in order to divide parallel, but not perpendicular, to the plane (Bergstralh et al., 2013). However, the identity of extrinsic molecular cues and their receptors on epithelial cells, which control this process, is largely unknown (Werts and Goldstein, 2011). Plexins comprise a family of transmembrane proteins that serve as receptors for semaphorins (Tamagnone et al., 1999). The semaphorin-plexin system represents a versatile intercellular signaling system both during development and in the adult organism. Semaphorin-plexin interactions have been shown to play central roles in cell-cell communication processes that control cell migration, proliferation, and differentiation in the nervous, immune, and bone systems (Gu and Giraudo, 2013; Kang and Kumanogoh, 2013; Koropouli and Kolodkin, 2014; Kumanogoh and Kikutani, 2013; Negishi-Koga and Takayanagi, 2012; Pasterkamp, 2012; Sakurai et al., 2012; Tamagnone, 2012; Worzfeld and Offermanns, 2014). In mammals, nine plexins have been identified, which are grouped into the following four subfamilies: Plexin-A1-4, Plexin-B1-3, Plexin-C1, and Plexin-D1. With high but incomplete penetrance, Plexin-B2-deficient mice display neural tube closure defects, which result in perinatal lethality (Deng et al., 2007; Friedel et al., 2007). Mice bypassing this phenotype show abnormalities in cerebellar granule cell migration as well as defects in corticogenesis and postnatal migration of neuroblasts in the subventricular zone (Deng et al., 2007; Friedel et al., 2007; Hirschberg et al., 2010; Saha et al., 2012).

Here, we uncover a previously unrecognized function of the semaphorin-plexin system as a crucial regulator of mitotic spindle orientation in dividing renal epithelial cells during morphogenesis and repair.

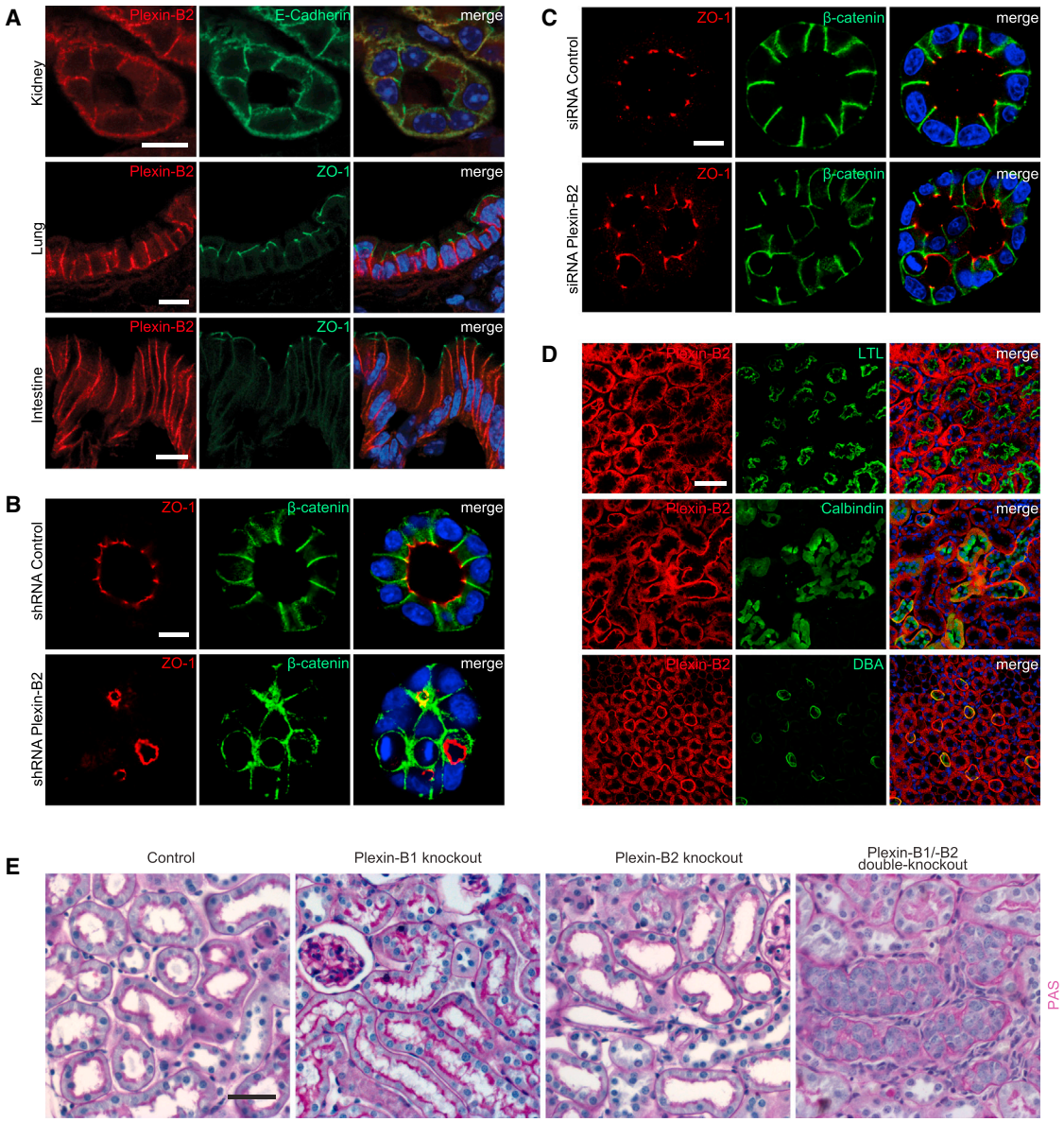


Figure 1. Plexins Are Required for Epithelial Morphogenesis

(A) Confocal images of immunostainings of adult mouse kidney, lung, and intestine using anti-Plexin-B2 (red) and anti-E-cadherin or anti-ZO-1 antibodies (green). Scale bars, 10 μm.

(B) MDCK cells stably expressing control or Plexin-B2 shRNA were grown in matrigel for 3 days. Shown are representative confocal images of immunostainings using anti-ZO-1 (red) and anti-β-catenin (green) antibodies. Scale bar, 10 μm.

(C) MDCK cells transfected with control or Plexin-B2 siRNA were grown in matrigel for 3 days. Shown are representative confocal images of immunostainings using anti-ZO-1 (red) and anti-β-catenin (green) antibodies. Scale bar, 10 μm.

(D) Confocal images of immunostainings of adult kidney sections using an anti-Plexin-B2 antibody (red) and the lectin LTL (green; marker of proximal tubules), an anti-Calbindin antibody (green; marker of distal tubules and collecting ducts), or the lectin DBA (green; marker of collecting ducts). Scale bar, 50 μm.

(E) Representative PAS stainings of adult control, Plexin-B1 single-deficient, Plexin-B2 single-deficient, and Plexin-B1/Plexin-B2 double-deficient kidneys. Scale bar, 50 μm.

RESULTS

Plexins Are Required for Epithelial Morphogenesis and Repair

We observed that Plexin-B2 is widely expressed in epithelial cells of adult mouse tissues in a strictly polarized manner and localizes to the basolateral compartment (Figure 1A). To investigate its functional role in epithelial cells, we employed the three-dimensional Madin-Darby canine kidney (MDCK) cell model, a well-established system to study epithelial morphogenesis. Consistent with its subcellular localization in epithelial cells in vivo, Plexin-B2 is co-expressed with markers of the

basolateral membrane also in MDCK cells in vitro (Figures S1A and S1B). Whereas MDCK cells stably expressing control short hairpin RNA (shRNA) formed mono-layered cysts with a single central lumen, MDCK cells transfected with Plexin-B2 shRNA were highly misorganized (Figures 1B, S1C, and S1D). Similar results were obtained when MDCK cells were transfected with a Plexin-B2 siRNA, the sequence of which was unrelated to the Plexin-B2 shRNA (Figures 1C, S1E, and S1F). In contrast to the striking abnormalities in three-dimensional MDCK cell cultures, we did not observe any defects in epithelial organization after knockdown of Plexin-B2 when MDCK cells were grown in two-dimensional monolayers (Figure S1G). To test whether Plexin-B2 is required for epithelial morphogenesis also in vivo, we chose the kidney as a model organ. A systematic analysis showed that all segments of the renal tubules strongly expressed Plexin-B2 (Figure 1D). Because of the perinatal lethality of mice with global inactivation of the Plexin-B2 gene, we inactivated the gene encoding Plexin-B2 specifically in renal tubular epithelial cells (TECs) by crossing a conditional allele for Plexin-B2 with the TEC-specific Pax8-Cre line (Pax8-Cre;*plxnb2*$^{flox/flox}$) (Figure S1H). Kidney morphology and function of mice with conditional inactivation of Plexin-B2 were comparable to their littermate controls (Figures 1E, 2F, 2G, S1I, and S1J). Of note, the closely related Plexin-B2 homolog Plexin-B1 is co-expressed with Plexin-B2 in the developing kidney, but is absent from adult TECs (Korostylev et al., 2008; Zielonka et al., 2010). Adult Plexin-B1 knockout mice were found to be devoid of morphological defects in the kidney (Figure 1E). To address a potential redundancy of Plexin-B1 and Plexin-B2, we generated double-deficient mice (Pax8-Cre;*plxnb1*$^{-/-}$;*plxnb2*$^{flox/flox}$). When analyzing adult kidney sections of these animals, we observed foci of multi-layered tubules (Figures 1E and S1K). These data suggest that plexin signaling is required for epithelial tissue morphogenesis when cells actively divide, but is dispensable in the mitotically quiescent renal epithelium at adult stages. We hypothesized that in the adult kidney, where Plexin-B1 is not expressed, cell division triggered by pathophysiological stimuli relies on Plexin-B2 signaling. To test this hypothesis in Plexin-B2-deficient mice, we employed a widely used mouse model of kidney repair, in which an ischemia/reperfusion (I/R) injury induces proliferation of surviving TECs within the plane of the epithelium to restore cell number and functional integrity (Bonventre and Yang, 2011). During the course of kidney repair, the expression pattern of Plexin-B2 in all segments of the renal tubules remained unchanged, whereas expression levels increased by almost 2-fold (Figures S2A and S2B). Kidney histology during the phase of acute organ damage 12 and 24 hr after injury was comparable between Plexin-B2-knockout and control animals, indicating that Plexin-B2 deficiency does not alter the susceptibility to injury (Figures 2A and S2C). Starting 3 days after injury, the tubular morphology in control animals progressively normalized, leading to full restoration 3 weeks after the ischemic insult (Figures 2A and S2C). In contrast, Plexin-B2-deficient TECs left the epithelial plane and intruded into the tubular lumen (Figures 2A and S2C), leading to a multi-layered epithelium and complete tubular occlusion by day 7 (Figures 2A, S2C, and S2D). Twenty-one days after ischemic injury, the generalized tubular abnormalities in Plexin-B2 knockout mice appeared highly reminiscent of the focal tubular abnormalities observed in uninjured kidneys of Plexin-B1/Plexin-B2 double knockouts (Figures 1E and 2A). The defects in epithelial architecture in Plexin-B2-deficient tubules after injury were accompanied by an increase in the rate of proliferation (Figures 2B and 2C), whereas apoptosis was unchanged (Figures 2D and 2E). To test whether the injury-induced changes in tissue architecture were also functionally relevant, we induced kidney injury bilaterally and determined serum creatinine and urea concentrations during kidney repair. Whereas the kidney function of control animals fully normalized within 3 weeks, Plexin-B2-deficient animals showed no signs of recovery, became moribund 7 days after injury, and had to be sacrificed (Figures 2F and 2G; data not shown).

Plexin-B2 Controls Mitotic Spindle Orientation in Epithelial Cells

We next investigated the cellular mechanisms responsible for the observed defects in epithelial architecture. We hypothesized that the loss of Plexin-B2-mediated signaling in renal TECs could result in misorientation of the mitotic spindle, with daughter cells being generated perpendicularly to the epithelial plane instead of parallel to it, thereby leading to multi-layering and luminar filling. This mechanism has been proposed for epithelial cancers where escape of cells from the epithelial sheet represents one of the early steps in tumorigenesis (McCaffrey and Macara, 2011). To test this hypothesis, we determined the mitotic spindle orientation in three-dimensional MDCK cysts. Whereas control MDCK cells mostly divided parallel to the epithelial plane, knockdown of Plexin-B2 shifted the cell division axis toward angles perpendicular to this plane (Figures 3A and 3B). We reasoned that if this shift of the mitotic spindle angle distribution was causative for the defects in epithelial architecture, then inhibition of cell division should result in recovery of normal epithelial architecture (Bañón-Rodríguez et al., 2014). Therefore, we analyzed how Plexin-B2 silencing affected MDCK cyst architecture when cells were maintained in the presence of thymidine, an inhibitor of mitosis. As expected, exposure of both control as well as Plexin-B2-silenced cells to thymidine led to smaller cyst diameters. Moreover, thymidine had no adverse effects on lumen formation and maintenance in control cells (Figures 3C and 3D) (Bañón-Rodríguez et al., 2014). Importantly, inhibition of mitosis of Plexin-B2-silenced cells by thymidine treatment resulted in recovery of normal lumina, corroborating that the abnormal architecture of Plexin-B2-silenced cysts is caused by misoriented cell division (Figures 3C and 3D). To study whether Plexin-B2 controls mitotic spindle orientation also in vivo, we analyzed kidneys 1 day after injury when renal TECs are just beginning to divide (Figure 2C) and before any defects in epithelial architecture become evident in Plexin-B2-deficient mice (Figures 2A and S2C). Indeed, while cells in control mice divided mostly within the epithelial plane, the mitotic spindle angle of Plexin-B2-deficient cells was shifted toward divisions perpendicular to the epithelial plane (Figures 3E and 3F). Also at 3 days after kidney injury, when Plexin-B2-deficient TECs are beginning to leave the epithelial sheet but epithelial architecture is still largely preserved (Figure 2A), a highly significant proportion of Plexin-B2-deficient TECs failed to align their divisions with the epithelial plane (Figures 3G and 3H). These data indicate that Plexin-B2 in dividing epithelial cells controls

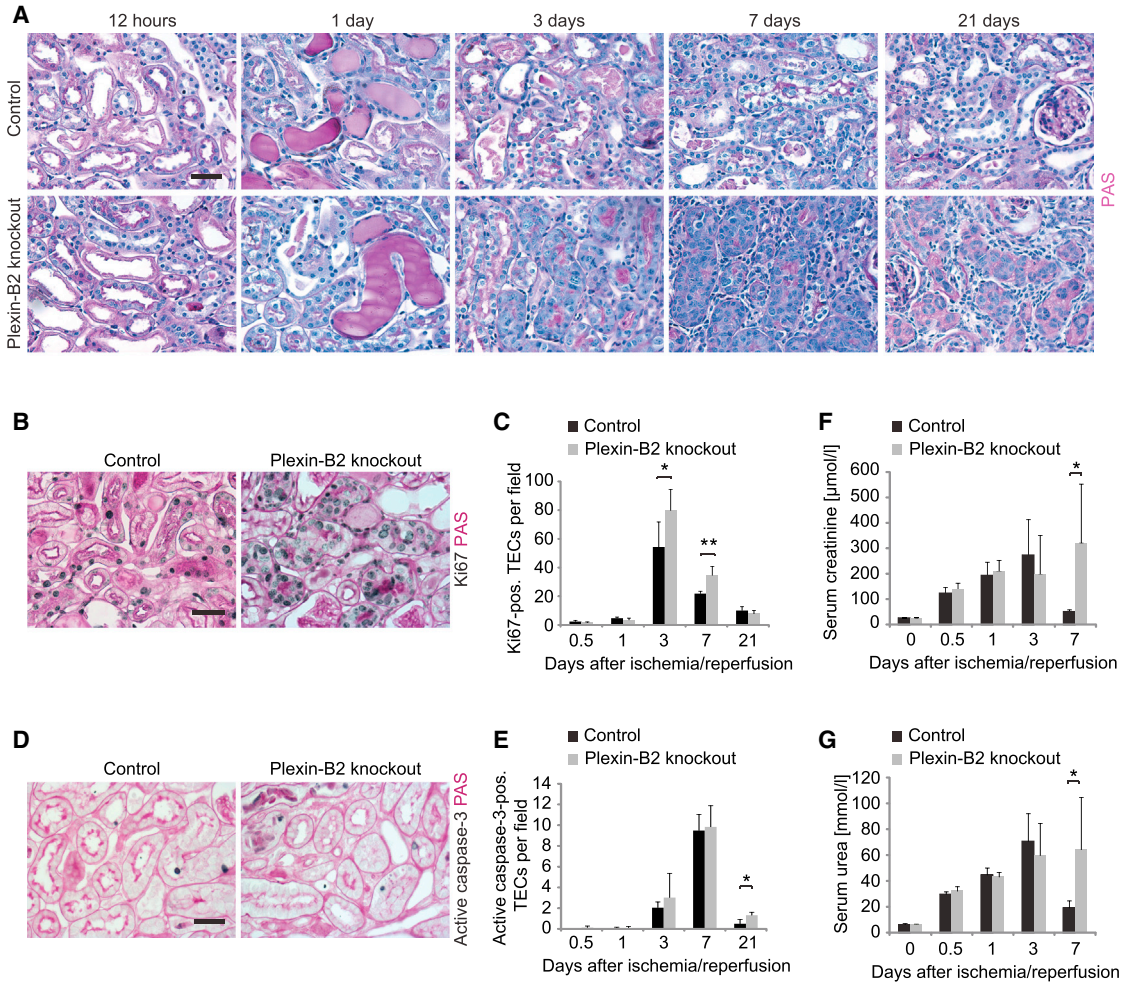


Figure 2. Plexin-B2 Is Essential for Kidney Repair

(A) PAS staining of kidney sections of control and Plexin-B2-deficient mice at the indicated time points after unilateral ischemia/reperfusion (I/R) injury. Scale bar, 50 µm.

(B) Representative pictures of anti-Ki67-immunostainings (gray) combined with PAS stainings of kidney sections of control and Plexin-B2-deficient mice 3 days after unilateral I/R injury. Scale bar, 50 µm.

(C) Quantification of Ki67-positive TECs per field (400×) after unilateral I/R injury at the indicated time points. Control mice: n = 4 for all time points; Plexin-B2 knockout mice: n = 4 at 12 hr, n = 5 at 1 day and 3 days, n = 7 at 7 days, and n = 4 at 21 days after I/R. Data are presented as mean ± SD.

(D) Representative pictures of anti-active caspase-3 immunostainings (gray) combined with PAS staining of kidney sections of control and Plexin-B2-deficient mice 3 days after unilateral ischemia/reperfusion injury. Scale bar, 50 µm.

(E) Quantification of anti-active caspase-3-positive TECs per field (400×) after unilateral I/R injury at the indicated time points. Control mice: n = 4 for all time points; Plexin-B2 knockout mice: n = 4 at 12 hr, n = 5 at 1 day and 3 days, n = 7 at 7 days, and n = 4 at 21 days after I/R. Data are presented as mean ± SD.

(F and G) Serum creatinine (F) and serum urea (G) levels in control and Plexin-B2-deficient mice before and after bilateral ischemia/reperfusion injury at the indicated time points. Control mice: n = 5 without injury, n = 4 at 12 hr and 1 day, n = 5 at 3 days, and n = 4 at 7 days after I/R; Plexin-B2 knockout mice: n = 5 without injury, n = 6 at 12 hr, n = 5 at 1 day, n = 6 at 3 days, and n = 4 at 7 days after I/R. Data are presented as mean ± SD.

epithelial architecture in vitro and in vivo through orientation of the mitotic spindle.

Semaphorins Signal through Plexin-B2 to Determine the Cell Division Axis

Plexin-B2 is activated by the semaphorins Sema4A, Sema4C, Sema4D, and Sema4G (Hirschberg et al., 2010; Maier et al., 2011; Masuda et al., 2004; Tamagnone et al., 1999; Yukawa et al., 2010). In a search for additional, previously unrecognized ligands, we identified Sema4B to also activate Plexin-B2 (Figure 4A). All these semaphorins were found to be expressed in the kidney both under physiological conditions as well as after injury (Figures S3A–S3C). Whereas Sema4B expression was detectable in TECs of all segments of the renal tubules, Sema4A and Sema4D exclusively localized to TECs of the proximal tubules (Figures 4C–4F). Within TECs, Sema4D, for which a specific antibody was available, localized to the basolateral membrane, similar to Plexin-B2 (Figure 4B). The expression pattern of these semaphorins did not change over the course of repair after kidney injury (Figures 4C–4F, S3A, and S3B). In contrast

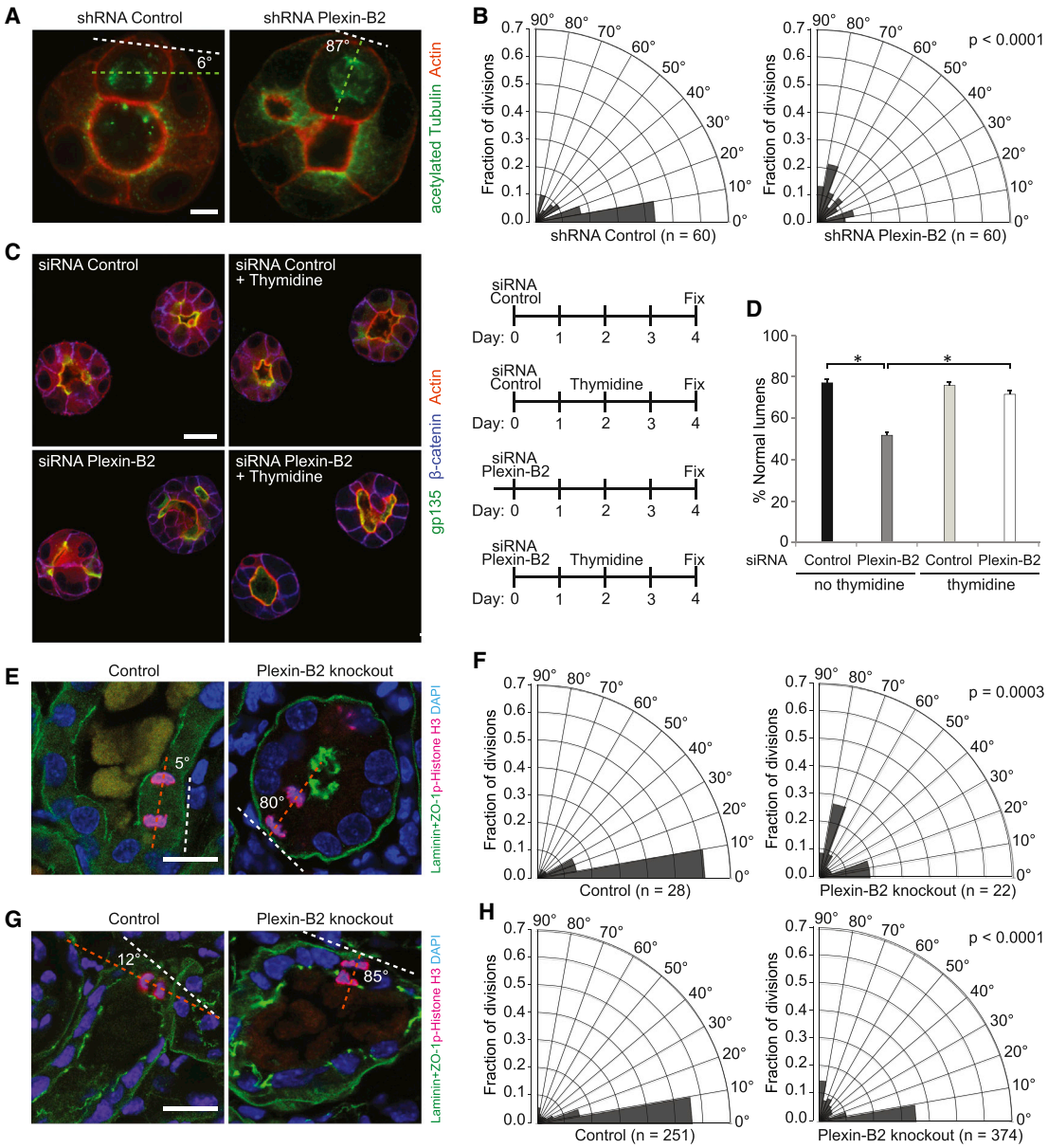


Figure 3. Plexin-B2 Controls Mitotic Spindle Orientation in Renal Epithelial Cells

(A) MDCK cells expressing control or Plexin-B2 shRNA were grown in matrigel for 3 days. Shown are examples of confocal images of immunostainings using phalloidin (red) and an anti-acetylated tubulin antibody (green). The epithelial plane is marked by a white dashed line, and the axis of mitosis is indicated by a green dashed line. The angle between these two lines represents the mitotic angle. Scale bar, 5 μm.

(B) Radial histograms (rose plots) depicting the mitotic spindle angle of dividing cells in MDCK cysts stably expressing control or Plexin-B2 shRNA at day 3 after embedding in matrigel.

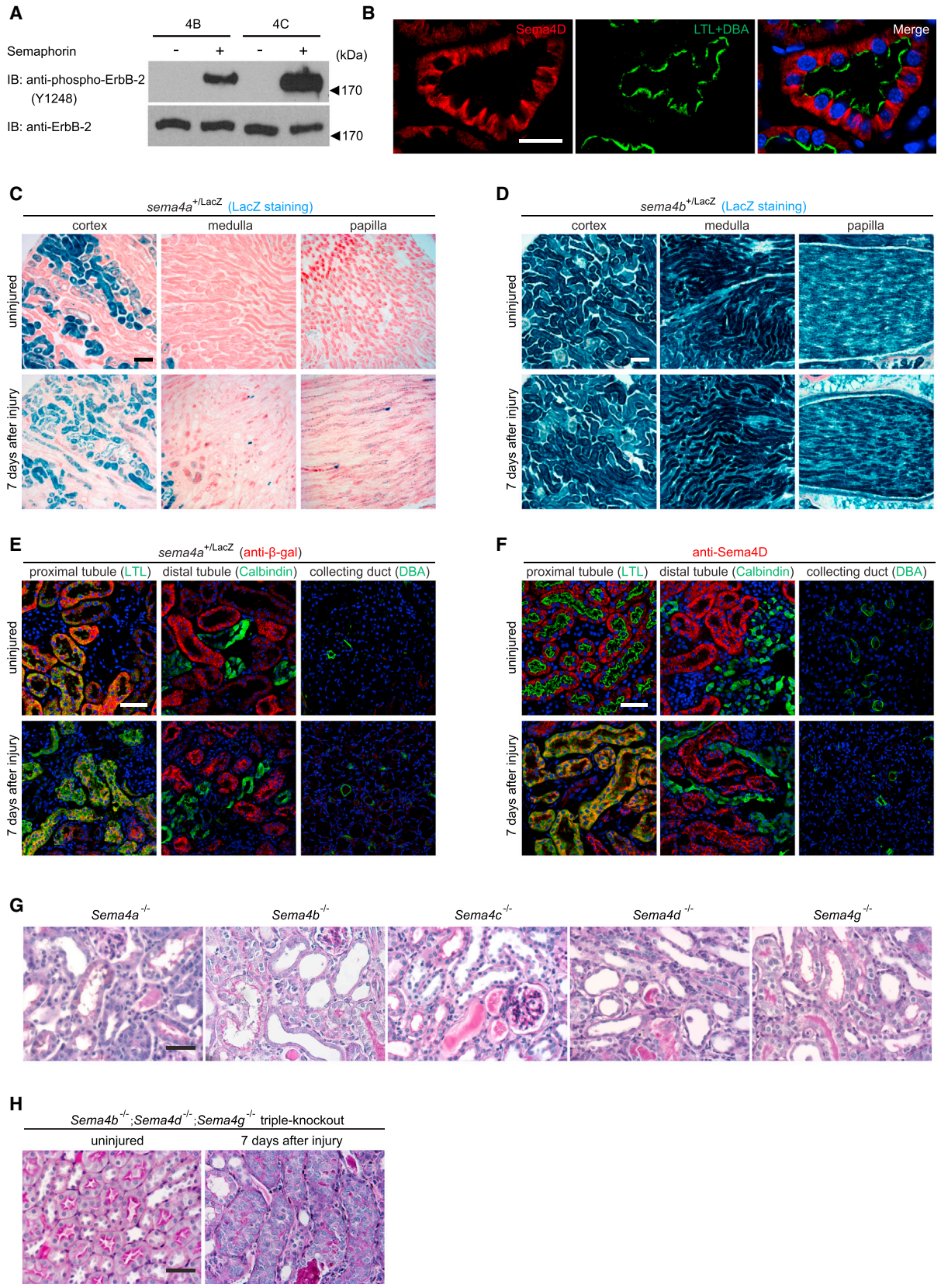
(C) MDCK cells were transfected with control or Plexin-B2 siRNA and grown in matrigel for 4 days. Starting at day 2, cysts were maintained in the presence or absence of the mitotic inhibitor, thymidine (2 mM). A scheme of the experimental design is depicted on the right. Shown are representative confocal images of immunostainings using anti-gp135 (green) and anti-β-catenin (blue) antibodies, and phalloidin to visualize actin (red). Scale bar, 20 μm.

(D) Quantification of the results shown in (C). Data are presented as mean ± SD.

(E and G) Examples of confocal images of immunostainings of renal tubules (E) 1 day or (G) 3 days after unilateral I/R injury using anti-phospho-histone H3 (red), anti-ZO-1 (green), and anti-Laminin-1 (green) antibodies. The plane of the tubular epithelium is marked by a white dashed line, and the axis of mitosis is indicated by a red dashed line. The angle between these two lines represents the mitotic angle. Scale bar, 25 μm.

(F and H) Radial histograms (rose plots) depicting the mitotic spindle angle of dividing cells of control and Plexin-B2-deficient TECs (F) 1 day or (H) 3 days after unilateral I/R injury; n = the total number of counted cells from three independent animals per group.

to Sema4A, Sema4B, and Sema4D, Sema4C was not expressed in TECs, but was in glomeruli and, after injury, also in interstitial cells (Figure S3A) (Zielonka et al., 2010). With the exception of Sema4G, which showed a complex temporal regulation of expression, the expression levels of all Plexin-B2 ligands were upregulated after kidney injury (Figure S3C). To test which of



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these semaphorins is the functionally relevant ligand of Plexin-B2 in the context of oriented cell division during kidney repair, we applied the I/R model to Sema4A-, Sema4B-, Sema4C-, Sema4D-, and Sema4G-deficient mice. All of these mice showed a normal kidney morphology under basal conditions (Figure S3D). Seven days after injury, none of the semaphorin knockouts phenocopied the Plexin-B2 knockout (Figure 4G). These results strongly suggest redundancy between the known semaphorin ligands of Plexin-B2 or the presence of an as yet unidentified semaphorin that activates Plexin-B2. To distinguish between these two scenarios, we focused on the combined genetic inactivation of the known semaphorin ligands of Plexin-B2. To this end, we generated triple-knockout mice lacking Sema4B, Sema4D, and Sema4G, which are expressed in renal tubular epithelial cells in vivo as well as in MDCK cells in vitro (Figures 4C–4F, S3A–S3C, and S4A). These mice were viable and displayed no overt defects in kidney histology under basal conditions (Figure 4H). Following ischemia/reperfusion injury, Sema4B/Sema4D/Sema4G triple-knockout mice phenocopied the misorganized, multi-layered tubular epithelium seen in Plexin-B2 knockout mice after injury (Figure 4H). These results indicate that Sema4B, Sema4D, and Sema4G act in concert to activate Plexin-B2 and to align the mitotic spindle axis with the epithelial plane. In contrast to Plexin-B2 knockout mice, in which all tubules of the entire renal cortex and outer medulla showed abnormal epithelial architecture after injury (Figure S4B), the tubular defects in Sema4B/Sema4D/Sema4G triple-knockout mice were confined to the outer renal medulla, whereas cortical tubules had a normal morphology (Figure S4C). This suggests the presence of another Plexin-B2 ligand in the renal cortex that maintains the correct orientation of cell divisions. Indeed, Sema4A localizes to tubular epithelial cells of this area (Figure S4D).

Plexin-B2 Signals through Its GAP Domain to Orient the Mitotic Spindle

Activation of Plexin-B2 by semaphorin ligands triggers the activation of intracellular signaling pathways that modulate the activity of small GTPases of the Ras and Rho family (Zhou et al., 2008) (Figure 5A). The intracellular domain of plexins shares homology with GTPase-activating proteins (GAPs) and confers the deactivation of the small GTPases R-Ras (Oinuma et al., 2004), M-Ras (Saito et al., 2009), and Rap1 (Wang et al., 2012). Via a stable interaction with RhoGEF proteins, Plexin-B2 also activates the small GTPase RhoA (Swiercz et al., 2002). To determine which Plexin-B2-mediated signaling pathways are required for correct orientation of cell division, we employed an allelic series of transgenic mice. In these animals, endogenous Plexin-B2 is replaced by transgenic Plexin-B2 versions carrying specific mutations in the intracellular domain (Worzfeld et al., 2014) (Figure 5A). Proper expression levels, expression pattern, and functionality of all transgenically expressed Plexin-B2 proteins have been extensively validated in various embryonic and adult tissues (Worzfeld et al., 2014). In addition, we confirmed that the expression of all transgenic Plexin-B2 proteins was equivalent to endogenous Plexin-B2 also in adult kidneys (Figures S5A and S5B). Mutant mice lacking the binding motif for RhoGEF proteins (ΔVTDL) showed normal kidney repair after I/R injury (Figure 5B), indicating that RhoA activation by Plexin-B2 is dispensable for mitotic spindle orientation. In contrast, transgenic mice carrying mutations in functionally relevant arginine residues of the Plexin-B2 GAP domain (R1395A, R1396G, R1691A) exhibited a multilayered and highly misorganized epithelium 7 days after I/R injury, indicating that proper alignment of the cell division axis with the plane of the epithelium depends on the GAP domain of Plexin-B2 (Figure 5C).

In addition to the modulation of the activity of small GTPases, semaphorin binding to B-subfamily plexins results in the transactivation and phosphorylation of the receptor tyrosine kinase, ErbB-2 (Swiercz et al., 2004). In HEK293 cells in vitro, Plexin-B2 versions lacking the intracellular part or carrying mutations in the critical arginines of the GAP domain could still activate ErbB-2 signaling to a comparable extent as the wild-type protein (Figure S5C). We next assessed the levels of ErbB-2 phosphorylation in kidney tissue and found that ErbB-2 phosphorylation was reduced in both uninjured and injured Plexin-B2-deficient kidneys when compared with controls (Figures S5D and S5E). This indicates that Plexin-B2-mediated transactivation of ErbB-2 also occurs in vivo and suggests that it could contribute to epithelial repair after kidney injury.

Cdc42 Is an Effector of Plexin-B2 in Oriented Cell Division

Epithelial morphogenesis depends on a highly interconnected and intricately regulated signaling network, in which small GTPases, particularly of the Ras and Rho subfamilies, play pivotal roles (Datta et al., 2011; Iden and Collard, 2008; Rodriguez-Boulan and Macara, 2014). The Rho GTPase Cdc42 has been shown to be crucial for correct spindle orientation both in epithelial cells in vitro (Bray et al., 2011; Jaffe et al., 2008;

Figure 4. Semaphorins Activate Plexin-B2 to Orient Cell Division

(A) Plexin-B2 interacts with the receptor tyrosine kinase ErbB-2. Activation of Plexin-B2 by semaphorin ligands results in the transactivation and tyrosine phosphorylation of ErbB-2. HEK293 cells, which endogenously express Plexin-B2 and ErbB-2, were incubated for 20 min with a conditioned medium of HEK293 cells expressing soluble Sema4B or Sema4C, cells were lysed, and lysates were immunoblotted using anti-phospho-ErbB-2 and anti-ErbB-2 antibodies.

(B) Confocal images of immunostainings of the adult kidney using the lectins LTL and DBA (green) and an anti-Sema4D antibody (red). Scale bar, 15 μm.

(C and D) LacZ stainings of kidney sections of (C) *sema4a*$^{+/lacZ}$ and (D) *sema4b*$^{+/lacZ}$ transgenic mice expressing β-galactosidase under the control of the respective endogenous promoters without injury or 7 days after unilateral ischemia/reperfusion injury. Scale bar, 100 μm.

(E) Confocal images of immunostainings of kidney sections derived from mice expressing β-galactosidase under the control of the Sema4a promoter (*sema4a*$^{+/lacZ}$) without injury or 7 days after unilateral ischemia/reperfusion injury using an anti-β-galactosidase antibody (red) and the lectin LTL (green; marker of proximal tubules), an anti-Calbindin antibody (green; marker of distal tubules and collecting ducts), or the lectin DBA (green; marker of collecting ducts). Scale bar, 50 μm.

(F) Confocal images of immunostainings of kidney sections of wild-type mice without injury or 7 days after unilateral ischemia/reperfusion injury using an anti-Sema4D antibody (red) and the lectin LTL (green), an anti-Calbindin antibody (green) or the lectin DBA (green). Scale bar, 50 μm.

(G) PAS staining of kidney sections of mice with the indicated genotypes 7 days after unilateral I/R injury. Scale bar, 50 μm.

(H) PAS staining of kidney sections of *Sema4b*$^{-/-}$;*Sema4d*$^{-/-}$;*Sema4g*$^{-/-}$ triple-knockout mice without injury or 7 days after unilateral I/R injury. Scale bar, 50 μm.

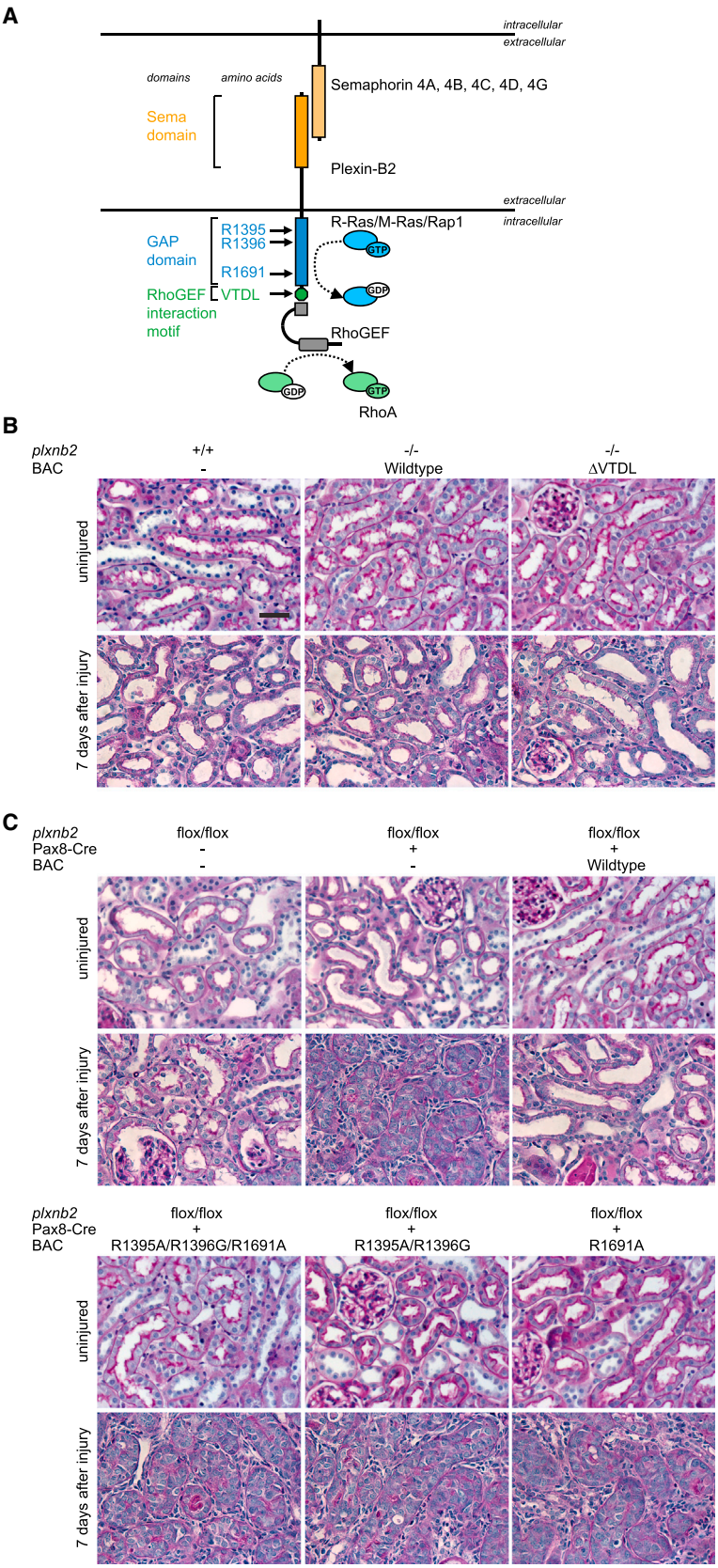


Figure 5. Plexin-B2 Signals through Its GAP Domain to Orient the Cell Division Axis

(A) Schematic illustration of major domains, functionally critical amino acids, and signaling pathways of Plexin-B2.

(B and C) PAS staining of kidney sections of mice with the indicated genotypes without injury or 7 days after unilateral I/R injury. Scale bar, 50 μm.

Mitsushima et al., 2009; Qin et al., 2010; Rodriguez-Fraticelli et al., 2010) as well as during neural and lung development in vivo (Kieserman and Wallingford, 2009; Wan et al., 2013). Since silencing of Cdc42 in MDCK cells results in a similar phenotype as silencing of Plexin-B2 (Martin-Belmonte et al., 2007), we hypothesized that Plexin-B2 orients the mitotic spindle by controlling Cdc42 activity. Indeed, Cdc42 activity was dramatically reduced in MDCK cysts following knockdown of Plexin-B2 (Figure 6A). To test whether Cdc42 activity was regulated by Plexin-B2 signaling also in vivo, we analyzed kidneys 3 days after ischemia/reperfusion injury. Consistent with the findings in MDCK cells, levels of active Cdc42 were markedly decreased in Plexin-B2-deficient kidneys as compared to controls (Figure 6B). To better characterize the link between Plexin-B2 signaling and Cdc42, we asked whether the modulation of the activity of R-Ras, M-Ras, or Rap1 downstream of Plexin-B2 impacts Cdc42 activity. Given that Rap1 is a critical regulator of Cdc42 activity (Iden and Collard, 2008), Rap1 appeared to be the most likely candidate. Unexpectedly, expression of constitutively active R-Ras, but not of constitutively active Rap1A, restored normal Cdc42 activity levels in Plexin-B2-silenced HEK293 cells (Figure 6C). We next investigated whether the regulation of Cdc42 activity by Plexin-B2 signaling was functionally relevant for the control of cell division orientation. Indeed, expression of constitutively active Cdc42 rescued the defects in MDCK cyst formation induced by Plexin-B2 silencing (Figures 6D and 6E). If these in vitro findings applied to the Plexin-B2-dependent regulation of the cell division axis in vivo, Cdc42 deficiency would result in similar defects as Plexin-B2 deficiency. We therefore analyzed the loss-of-function phenotype of Cdc42 in kidney repair using a doxycycline-inducible kidney-specific knockout mouse line. In contrast to mice with a constitutive, kidney-specific inactivation of Cdc42 at embryonic stages, which develop cystic kidneys and die from renal failure within weeks after birth (Choi et al., 2013), mice with doxycycline-induced inactivation of Cdc42 at adult stages displayed normal kidney histology and function under basal conditions (Figures 6F and 6H). Following ischemia/reperfusion injury, however, these mice exhibited a misorganized, multi-layered, hyperproliferative epithelium and impaired recovery of renal function, similar to mice lacking Plexin-B2 and mice expressing Plexin-B2 arginine mutants (Figures 6F–6J). Like Plexin-B2-deficient cells, dividing Cdc42-deficient cells during kidney repair showed a highly significant shift toward mitotic spindle angles perpendicular to the epithelial plane (Figures 6K and 6L). Collectively, these data establish Cdc42 as a downstream effector of Plexin-B2 in the control of the cell division axis.

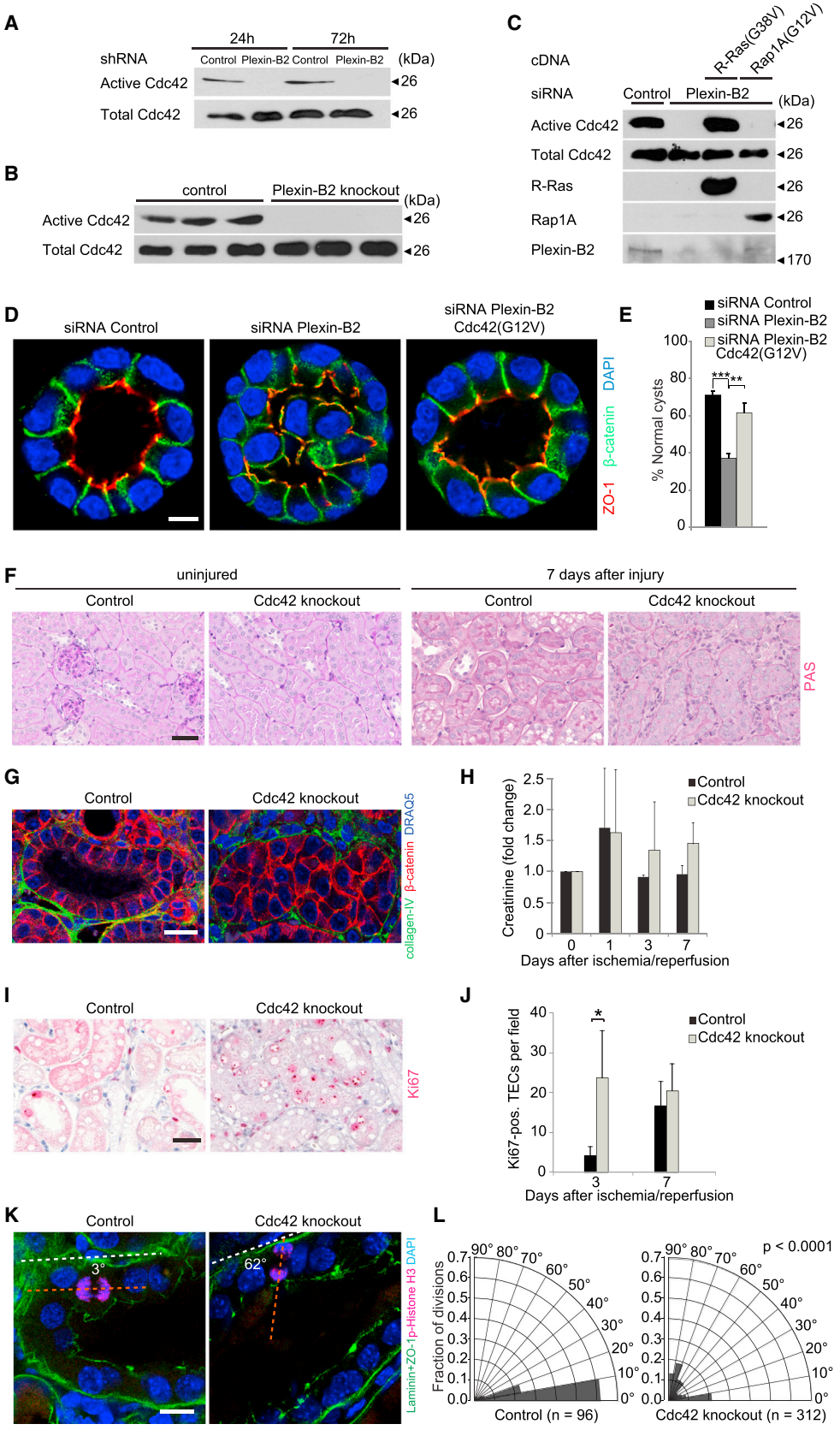
DISCUSSION

The intracellular molecular pathways that align the mitotic spindle of symmetrically dividing epithelial cells with the epithelial plane have been extensively studied (Lu and Johnston, 2013; Schlüter and Margolis, 2012). However, extracellular cues and their receptor systems that control these intracellular signaling networks are largely unknown. Here, we have identified the semaphorin-plexin system to regulate the cell division axis. While cell division orientation has so far been analyzed mainly in the context of epithelial morphogenesis in developing organisms, our data show that correct alignment of the mitotic spindle axis is also essential for epithelial repair processes.

In the adult kidney during repair, the combined genetic inactivation of the semaphorins Sema4B, Sema4D, and Sema4G; the genetic inactivation of their receptor Plexin-B2; the replacement of endogenous Plexin-B2 with transgenic Plexin-B2 versions carrying mutations in the GAP domain; or the genetic inactivation of Cdc42 resulted in misoriented cell divisions and severe defects in epithelial architecture of renal tubules. Our data are consistent with a model in which homotypic cell-cell-communication between epithelial cells provides essential spatial information to correctly orient the mitotic spindle. The semaphorins 4B, 4D, and 4G expressed at the membrane of tubular epithelial cells act as positional cues that are sensed by neighboring epithelial cells expressing the receptor Plexin-B2. Activated Plexin-B2 then signals through its GAP domain and Cdc42 to align cell divisions with the epithelial plane. Given that renal tubular epithelial cells co-express semaphorins and Plexin-B2, this signaling could potentially occur bidirectionally, with each epithelial cell both providing as well as receiving spatial information through the same ligand-receptor system.

Our data indicate that during epithelial repair, lack of a single semphorin can be compensated for by other semaphorins of the same subfamily. In line with this, studies in the nervous system have also reported redundancy between different semaphorins. For example, Sema5A and Sema5B cooperate in regulating retinal lamination, and Sema4C and Sema4G act in concert to control cerebellar granule cell migration (Maier et al., 2011; Matsuoka et al., 2011). In contrast to epithelial repair, cell division orientation during kidney development was not affected in Sema4B/Sema4D/Sema4G triple-deficient deficient mice, and Plexin-B1/Plexin-B2 double deficiency led to malformation of some, but not all, kidney tubules. These data strongly suggest an even higher degree of redundancy among semaphorins and plexins during kidney development or the presence of additional ligand-receptor systems that control the alignment of the mitotic spindle with the epithelial plane. It seems likely that because of redundancies among semaphorins and plexins, important functions of this signaling system in physiology and disease have gone unnoticed so far. Recent evidence showing that plexins can be activated by not only semaphorins but also ligands of the Slit family adds an additional level of complexity and potentially opens the possibility for compensations even across different ligand families (Delloye-Bourgeois et al., 2014).

Surprisingly, renal tubular epithelial cells in semaphorin, Plexin-B2, and Cdc42 mutant mice that divided perpendicularly to the epithelial plane into the tubular lumen did not undergo anoikis following delamination from the basal lamina (Taddei et al., 2012). Interestingly, it has been reported recently that in developing kidneys, tip cells of the ureteric bud frequently divide inside the lumen of the ureteric bud (Packard et al., 2013). While one of the daughter cells typically retains contact to the basal lamina by extending a thin cellular process, the other daughter cells transiently delaminates from the basal lamina without undergoing anoikis. Both daughter cells then reintegrate into the epithelial cell layer, resulting in frequent cell rearrangements (Packard et al., 2013). In contrast to this physiological process, delaminated cells in semaphorin, plexin, or Cdc42 mutant mice did not reintegrate but remained in the lumen to form a



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multi-layered epithelium. Moreover, the misorientation of the cell division axis in epithelial cells of these mutant mice was accompanied by hyperproliferation, resulting in a "tumor-like" appearance of the tubules. Of note, we did not observe any cells to cross the basal lamina. Given that the activity of the receptor tyrosine kinase ErbB-2, which promotes proliferation and luminal filling (Muthuswamy et al., 2001) and prevents anoikis of epithelial cells (Reginato et al., 2003), is reduced in Plexin-B2-deficient kidneys, other signaling mechanisms must account for these effects. It is well-established in stem cells that misalignment of the mitotic spindle axis can result in a shift from asymmetrical to symmetrical division leading to hyperproliferation through expansion of the stem cell pool (Noatynska et al., 2012). However, stem cells do not substantially contribute to kidney repair (Humphreys et al., 2008). Whether the hyperproliferation of Plexin-B2- and Cdc42-deficient TECs is a consequence of the deviations of the mitotic spindle axis or an independent process remains to be determined.

Although Cdc42 has been established as a central regulator of mitotic spindle orientation (Bray et al., 2011; Jaffe et al., 2008; Kieserman and Wallingford, 2009; Mitsushima et al., 2009; Wan et al., 2013), little is known about regulatory proteins that control the activity of Cdc42 at the plasma membrane (Lu and Johnston, 2013; Martin-Belmonte and Mostov, 2008). Here, we show that loss of Plexin-B2 abolishes Cdc42 activity in MDCK cells in vitro and in injured kidneys in vivo,and that mice lacking Cdc42 phenocopy the spindle orientation defects of Plexin-B2 mutant mice. The misorientation of the mitotic spindle was stronger in Cdc42-deficient than in Plexin-B2-deficient renal epithelial cells, arguing for the existence of additional signaling pathways controlling Cdc42 activity. Given that Rap1 is both a downstream effector of Plexin-B2 and a known upstream regulator of Cdc42 activity (Iden and Collard, 2008; Wang et al., 2012), Rap1 seemed likely to provide a molecular link from Plexin-B2 to Cdc42. However, our data suggest that Plexin-B2 signals to Cdc42 via R-Ras, rather than via Rap1. To our knowledge, R-Ras has not been implicated in the regulation of Cdc42 activity so far. In yeast and other fungi, a Ras1-Cdc42 pathway has been shown to control the coordination of polarized cell growth (Chang and Philips, 2006), and in rodent and human fibroblasts, signaling from H-Ras to Cdc42 has been reported to be crucial for oncogenic transformation (Arias-Romero and Chernoff, 2013). In these systems, the Ras-mediated regulation of Cdc42 activity occurs at endomembranes and seems to involve the recruitment or activation of Cdc42 GEFs by Ras. Additional studies will be required to clarify whether similar molecular mechanisms also apply to the regulation of Cdc42 by the Ras isoform, R-Ras.

In addition to signaling via its intracellular domain, Plexin-B2 can also signal via its extracellular domain to transactivate the receptor tyrosine kinases (RTKs), ErbB-2 and Met (Giordano et al., 2002; Swiercz et al., 2004; unpublished data), both of which are known to be centrally involved in epithelial morphogenesis and repair processes, particularly in the mammary gland, the skin, and the liver (Gherardi et al., 2012; Jackson-Fisher et al., 2004). The finding that the activity of ErbB-2 was reduced in uninjured Plexin-B2-deficient kidneys reveals a tonic activation of semaphorin-Plexin-B2 signaling under physiological conditions without an ischemic insult. While our data using transgenic mice, which carry mutations in critical arginines of the Plexin-B2 GAP domain, show clearly that signaling through the intracellular domain of Plexin-B2 is essential for correct orientation of the cell division axis and kidney repair, the decreased activity of ErbB-2 before and after injury could additionally contribute to the observed phenotype. Given that the RTK, Met, confers protective effects on renal tubular epithelial cells, rather than to promote repair (Zhou et al., 2013), transactivation of Met by

Figure 6. Cdc42 Is a Downstream Effector of Plexin-B2 in Mitotic Spindle Orientation

(A) MDCK cells stably expressing control or Plexin-B2 shRNA were grown in matrigel for 24 or 72 hr. After cell lysis, the amounts of active Cdc42 were determined (pulldown).

(B) 3 days after ischemia/reperfusion injury, kidneys of control or Plexin-B2-deficient mice were lysed and the amounts of active Cdc42 were determined (pulldown). Each lane represents a kidney lysate of an independent mouse.

(C) HEK293 cells were transfected with control or Plexin-B2 siRNA and with cDNAs encoding constitutively active myc-tagged R-Ras (R-Ras (G38V)) or constitutively active HA-tagged Rap1A (Rap1A(G12V)) where indicated. After 24 hr, cells were lysed and the amounts of active Cdc42 were determined (pulldown). In addition, expression levels of Cdc42, R-Ras (using an anti-myc antibody), Rap1A (using an anti-HA antibody), and Plexin-B2 were analyzed in cell lysates.

(D) MDCK cells expressing constitutively active myc-tagged Cdc42(G12V) under the control of the tet-off repressor were transfected with control or Plexin-B2 siRNA and allowed to form cysts for 72 hr in the presence of doxycycline (20 ng/ml; Cdc42(G12V) expression repressed) or absence of doxycycline (Cdc42(G12V) expression induced). Shown are representative confocal images of immunostainings using anti-ZO-1 (red) and anti-β-catenin (green) antibodies. Scale bar, 10 μm.

(E) Quantification of the results shown in (D). Data are presented as mean ± SD.

(F) PAS staining of kidney sections of control and Cdc42-deficient mice without injury or 7 days after unilateral I/R injury. Scale bar, 50 μm.

(G) Confocal images of immunostainings of adult kidney sections 7 days after unilateral ischemia/reperfusion injury using anti-collagen-IV (green) and anti-β-catenin (red) antibodies. Scale bar, 20 μm.

(H) Serum creatinine levels in control and Cdc42-deficient mice after bilateral ischemia/reperfusion injury at the indicated time points. Control mice: n = 3; Cdc42 knockout mice: n = 3. Data are presented as mean ± SD.

(I) Representative pictures of anti-Ki67-immunostainings (red) of kidney sections of control and Cdc42-deficient mice 3 days after unilateral I/R injury. Scale bar, 50 μm.

(J) Quantification of Ki67-positive TECs per field (400×) after unilateral I/R injury at the indicated time points. Control mice: n = 5 at 3 days and n = 3 at 7 days after I/R; Cdc42 knockout mice: n = 3 at 3 days and n = 3 at 7 days after I/R. Data are presented as mean ± SD.

(K) Examples of confocal images of immunostainings of renal tubules 3 days after unilateral I/R injury using anti-phospho-histone H3 (red), anti-ZO-1 (green), and anti-Laminin-1 (green) antibodies. The plane of the tubular epithelium is marked by a white dashed line, and the axis of mitosis is indicated by a red dashed line. The angle between these two lines represents the mitotic angle. Scale bar, 10 μm.

(L) Radial histograms (rose plots) depicting the mitotic spindle angle of dividing cells of control and Cdc42-deficient TECs 3 days after unilateral I/R injury; n = the total number of counted cells from three independent animals per group.

Plexin-B2 seems unlikely to be relevant after ischemia/reperfusion injury. To test for a functional involvement of Plexin-B2-mediated transactivation of RTKs in mitotic spindle alignment and kidney repair, specific mutations in Plexin-B2 or anti-Plexin-B2 antibodies, which interfere with the transactivation of receptor tyrosine kinases but leave the binding of semaphorin ligands and the activation of Plexin-B2 intracellular signaling unaffected, would be required. However, these tools are not available to date.

While the semaphorin-plexin system had not been implicated in oriented cell division so far, semaphorins and plexins have been shown to be involved in kidney organogenesis and disease. In the developing kidney, different semaphorins and plexins serve as negative or positive regulators of ureteric bud branching morphogenesis, modulate podocyte differentiation, and control podocyte-endothelial crosstalk to establish and maintain a normal glomerular filtration barrier (Reidy and Tufro, 2011). Following ischemia/reperfusion injury, the expression of Sema3A increases in podocytes and tubular epithelial cells (Ranganathan et al., 2014). Exogenous application or podocyte-specific overexpression of Sema3A induces proteinuric glomerular disease; conversely, global inactivation of Sema3A protects from ischemia/reperfusion-induced injury (Ranganathan et al., 2014; Tufro, 2014). These data combined with our findings indicate that whereas particular semaphorins, namely Sema3A, promote kidney injury by causing podocyte damage, aggravating kidney inflammation, and increasing apoptosis of tubular epithelial cells, others (i.e., class 4 semaphorins) exert protective effects in the injured kidney by ensuring maintenance of epithelial architecture during repair. Several experimental studies identified Sema3A in the urine as an early predictive biomarker of acute kidney injury both in mouse models and in human patients (Jayakumar et al., 2013), and a clinical trial assessing the potential use of Sema3A as a biomarker has been registered (the University Hospital Medical Information Network Clinical Trials Registry [UMIN-CTR] trial number UMIN000013422). We found that, similar to Sema3A, expression levels of several class 4 semaphorins in renal tubular epithelial cells were upregulated after acute kidney injury. Unlike class 3 semaphorins, which are secreted, soluble molecules, class 4 semaphorins are transmembrane proteins. However, several class 4 semaphorins have been reported to be released from the cell surface by regulated proteolysis (Elhabazi et al., 2001; Meissner et al., 2013). Whether class 4 semaphorins become detectable in the urine after kidney injury and could potentially serve as biomarkers warrants further investigation.

In summary, we identified a plexin receptor on epithelial cells that controls the alignment of the cell division axis with the epithelial plane in a semaphorin-dependent manner by signaling through its GAP domain and Cdc42. It is tempting to speculate that regulation of cell division orientation by the semaphorin-plexin signaling system could also be instrumental in other tissues under both physiological and pathophysiological conditions including cancer.

EXPERIMENTAL PROCEDURES

Mice

To generate mice lacking Plexin-B2 specifically in the kidney, mice carrying a conditional allele for Plexin-B2 (Deng et al., 2007) were crossed with mice expressing Cre under the control of the Pax8 promoter (Bouchard et al., 2004) (Pax8-Cre;*plxnb2*$^{flox/flox}$). The generation of BAC transgenic mice expressing MYC-tagged wild-type Plexin-B2 or MYC-tagged Plexin-B2 versions harboring specific mutations has been described previously (Worzfeld et al., 2014). The Plexin-B1 knockout allele used to generate Plexin-B1/Plexin-B2 double-deficient mice (Pax8-Cre;*plxnb1*$^{-/-}$;*plxnb2*$^{flox/flox}$) has also been described previously (Deng et al., 2007). Mice carrying a conditional allele of Cdc42 (*cdc42*$^{flox/flox}$) (Wu et al., 2006) were crossed with LC1-Cre mice expressing Cre under the control of the P_{tet} promoter (LC1-Cre) (Schönig et al., 2002), and with Pax8-rtTA mice expressing a reverse tetracycline-dependent transactivator (rtTA) under the control of the Pax8 promoter (Pax8-rtTA) (Traykova-Brauch et al., 2008), to generate mice with an inducible, kidney-specific deletion of Cdc42 (LC1-Cre;Pax8-rtTA;*cdc42*$^{flox/flox}$). *Cdc42*$^{flox/flox}$ mice served as controls. To both LC1-Cre;Pax8-rtTA;*cdc42*$^{flox/flox}$ and *cdc42*$^{flox/flox}$ animals, doxycycline was administered for 3 weeks at a concentration of 0.2 mg ml^{-1} in the drinking water supplemented with 2.5% sucrose prior to ischemia/reperfusion injury. ES cells carrying a targeted allele of the Sema4A gene were obtained from EUCOMM (allele name Sema4a$^{tm1a(EUCOMM)Wtsi}$, clone ID EPD0105_3_B05). Correct targeting was verified by Southern blot. Following blastocyst injection, chimeras were mated with C57Bl/6 wild-type mice, and germline transmission of the targeted allele was achieved. The resulting mouse line was used for expression analyses of Sema4A (*sema4a*$^{+/LacZ}$) or crossed with E2A-Cre to remove floxed sequences. After mating to homozygosity, the obtained null allele (*sema4a*$^{-/-}$) was used for functional analyses. The *sema4b* and *sema4c* genes were mutated by gene trapping and targeted trapping, respectively, resulting in functional null alleles and expression of β-galactosidase under the control of the respective endogenous promoters (Maier et al., 2011). Sema4D and Sema4G knockout mice were generated as described (Kumanogoh et al., 2000; Maier et al., 2011). All mouse lines used in the study were kept on a C57BL/6 background (backcrossed for at least seven generations). All procedures of animal care and use in this study were approved by the local animal ethics committee (Regierungspräsidium Darmstadt, Germany).

Ischemia/Reperfusion Kidney Injury Model

Adult male mice (age between 10 and 20 weeks) were anesthetized with isofluorane. The kidney was accessed retroperitoneally and the renal pedicle was exposed, carefully isolated from surrounding fat tissue, and clamped with a micro-serrefine (Fine science tools, 18055-05). For unilateral I/R, the clamp was placed on the left renal pedicle for 30 min, while for bilateral I/R both renal pedicles were clamped for 25 min. During ischemia/reperfusion surgery, mice were kept on a heating pad and body temperature was maintained at 37°C. Afterward, clamps were removed and the incision was closed. For all experiments using Plexin-B2 or Cdc42 knockout mice, Cre-negative *plxnb2*$^{flox/flox}$ or *cdc42*$^{flox/flox}$ littermates served as controls. The semaphorin knockout mouse lines (Sema4A KO, Sema4B KO, Sema4C KO, Sema4D KO, Sema4G KO, Sema4B/Sema4D/Sema4G triple-KO) and the Plexin-B2 BAC transgenic mouse lines (BAC Plexin-B2 wild-type, BAC Plexin-B2 [ΔVTDL], BAC Plexin-B2 [R1395A/R1396G/R1691A], Plexin-B2 [R1395A/R1396G], and Plexin-B2 [R1691A]) were bred as separate colonies.

Analysis of Mitotic Spindle Angle

In MDCK cells, the mitotic spindle angle was determined as described (Bañón-Rodríguez et al., 2014). Kidney sections 1 day or 3 days after ischemia/reperfusion injury were immunostained for ZO-1 and Laminin-1 to visualize the apical and basement membrane, respectively. Dividing cells were marked by immunoreactivity for phosphorylated histone H3 (Ser10). The angle of cell division was determined by measuring the angle between the plane of mitosis (axis transecting two daughter nuclei) and the plane of the basement membrane using Adobe Photoshop software. The analysis of the mitotic spindle angle was performed by an observer blinded to mouse genotypes. Statistical significance was determined by the Mann-Whitney U test on pooled data of three independent mice per genotype. Radial histograms (rose plots) were generated using OpenStereo software (http://www.igc.usp.br/index.php?id=395).

Determination of Activated Cdc42

6-cm dishes were coated with 100 μl cold matrigel, and 4 × 10^5 MDCK cells in 2% matrigel/culture medium were plated. Cells were cultured for 24 or 72 hr

(after 48 hr, the medium was removed and replaced by fresh 2% matrigel/culture medium), scraped off, and lysed in a 500 μl ice-cold pulldown buffer (50 mM Tris [pH 7.2], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, and protease inhibitors). Lysates were immediately centrifuged for 10 min at 4°C, and supernatants were incubated with a fusion protein consisting of GST and the Cdc42-binding domain of PAK (GST-PAK) coupled to agarose beads for 50 min at 4°C. Beads were then washed three times with an ice-cold pulldown buffer, boiled with a Laemmli buffer for 5 min, and subjected to western blotting. The amount of activated Cdc42 in HEK293 cells, which were grown on uncoated 6-cm dishes, was determined analogously. For the determination of activated Cdc42 in mouse kidneys 3 days after injury, kidneys were homogenized in an ice-cold pulldown buffer, and GTP-bound Cdc42 was precipitated using GST-PAK as described above.

Statistical Analysis

Statistical significances were evaluated by two-tailed t tests with error bars representing standard deviations (Figures 2C, 2E–2G, 3D, 6E, 6H, 6J, S1A, S1C–S1F, S1K, S2B, S3C, S5E, and S6A) and Mann-Whitney U tests (Figures 3B, 3F, 3H, and 6L). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.02.001.

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