Slp2-a controls renal epithelial cell size through regulation of Rap–ezrin signaling independently of Rab27

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Running title: Slp2-a controls renal epithelial cell size

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Summary

Slp2-a is a Rab27 effector protein that regulates transport of Rab27-bearing vesicles/organelles via its N-terminal Rab27-binding domain and a phospholipid-binding C2A domain. Here we demonstrate a Rab27-independent function of Slp2-a in the control of renal cell size via a previously uncharacterized C2B domain. We found that by recruiting Rap1GAPs to the plasma membrane of MDCK II cells via the C2B domain Slp2-a inactivates Rap signaling and modulates the size of the cells. Functional ablation of Slp2-a resulted in an increase in the size of MDCK II cells. *Drosophila* Slp bitesize was found to compensate for the function of Slp2-a in MDCK II cells, thereby indicating that the mechanism of the cell size control by Slps has been evolutionarily conserved. Interestingly, blockade of the activity of ezrin, a downstream target of Rap, with the glucosylceramide synthase inhibitor miglustat effectively inhibited cell spreading of Slp2-a-knockdown cells. We also discovered aberrant expression of Slp2-a and increased activity of ezrin in pcy mice, a model of polycystic kidney disease that is characterized by renal cell spreading. Our findings indicate that Slp2-a controls renal cell size through regulation of Rap–ezrin signaling independently of Rab27.

**Key words:** cell size, ezrin, polycystic kidney disease, Rap1GAP, Slp2-a
Introduction

Slp2-a is a member of the synaptotagmin-like protein (Slp) family, whose members consist of an N-terminal Slp homology domain (SHD) and C-terminal tandem C2 domains (named the C2A domain and C2B domain) (Fukuda et al., 2001; Fukuda and Mikoshiba, 2001), and it was originally characterized as an effector molecule for the small GTPase Rab27 (Fukuda, 2006; Kuroda et al., 2002). In melanocytes, Slp2-a is localized on mature melanosomes and promotes their anchoring to the plasma membrane through interaction with Rab27 on the melanosomes via the SHD and with phospholipids in the plasma membrane via its C2A domain (Kuroda and Fukuda, 2004). Similarly, Slp2-a promotes docking of secretory vesicles to the plasma membrane in certain types of secretory cells (Holt et al., 2008; Ménasché et al., 2008; Saegusa et al., 2006). Slp2-a has recently been shown to be localized at the apical membrane of renal epithelial cells and to be involved in the regulation of cell signaling and tubulogenesis by promoting docking of Rab27-bearing podocalyxin-containing vesicles to the apical membrane (Gálvez-Santisteban et al., 2012; Yasuda et al., 2012). Based on these findings, Slp2-a is generally thought to be a key mediator of the docking of Rab27-bearing vesicles/organelles to the plasma membrane, and the docking process is thought to require both the Rab27 binding activity of the SHD and the phospholipid binding activity of the C2A domain. In contrast to the C2A domain, however, the physiological function of the C2B domain, which exhibits only weak phospholipid binding ability (Gálvez-Santisteban et al., 2012; Kuroda and Fukuda, 2004), has yet to be determined.

Although the Rab27 effector function of Slp2-a has been well established in the
literature, several studies implicate a Rab27-independent role of Slp2-a in cell morphology. As an example, Slp2-a has been shown to be involved in generation of the elongated morphology of melanocytes, but the molecular mechanism by which it does so unknown (Kuroda and Fukuda, 2004). Another example is the involvement of the *Drosophila* Slp homologue bitesize (Btsz), the only homologue of mammalian Slp that shows high sequence similarity to Slp2-a (Fukuda et al., 2001), in maintenance of epithelial cell morphology (Pilot et al., 2006; Serano and Rubin, 2003), although the precise mechanism here also remains unknown. Because Btsz protein lacks an N-terminal SHD, i.e., a Rab27-binding domain, Rab27 effector function must not have been retained during evolution, and we hypothesized the existence of a conserved function of Slps in cell morphology.

In this study we investigated the possible involvement of Slp2-a in controlling the morphology of Madin-Darby canine kidney II (MDCK II) cells (a renal epithelial cell line) and showed that it regulates renal cell size through Rap–ezrin signaling by recruiting Rap1GAPs to the plasma membrane via the C2B domain. Because Slp2-a is also expressed in the renal tubules of mice (Yasuda et al., 2012), we investigated expression of Slp2-a in *pcy* mice, a spontaneous model of polycystic kidney disease (PKD), that have a homozygous missense mutation (I614S) in the *nephrocystin-3* (*NPHP3*) gene (Bergmann et al., 2008; Hildebrandt et al., 2009; Olbrich et al., 2003; Torres and Harris, 2009). Cystic kidney diseases, including autosomal dominant and autosomal recessive PKDs and nephronophthisis (NPHP), are a heterogeneous group of genetic disorders all of which are characterized by renal cell spreading, renal cyst growth, and progression to kidney failure (Hildebrandt et al, 2009; Torres and Harris, 2009; Wilson, 2004), but the precise mechanisms underlying the diseases are poorly
understood. We discovered aberrant expression of Slp2-a and increased activity of ezrin in pcy mice. Our findings indicate that Slp2-a-mediated control of renal cell size is crucial for normal kidney function, and we discuss Slp2-a-mediated Rap–ezrin signaling as a potential target for the treatment of PKD.
Results

Slp2-a regulates cell size in renal epithelial cells

First, we investigated the involvement of Slp2-a in renal cell proliferation by comparing the growth rates of control MDCK II cells and Slp2-a-knockdown (KD) MDCK II cells that we had established previously by using Slp2-a short hairpin RNA (shRNA) (Yasuda et al., 2012) (Fig. 1A,B). Although there was no difference in their growth rate, the Slp2-a-KD cells became confluent much faster than the control cells. We then investigated the morphology of Slp2-a-KD cells (Fig. 1C). Intriguingly, the Slp2-a-KD cells were significantly larger (~4 times larger) than the control cells under subconfluent conditions. Greater size of the Slp2-a-KD cells (~1.5 times larger than control cells) was observed even under polarized conditions (see Yasuda et al., 2012), but since their phenotype was not so clearly distinguishable as the phenotype of the subconfluent cells, we decided to use subconfluent MDCK II cells to investigate the role of Slp2-a in cell size control in the subsequent analyses. The greater size of the Slp2-a-KD cells was unlikely to have been attributable to an off-target effect of shRNA, because the same results were obtained by transient expression of three different Slp2-a small interfering RNAs (siRNAs) in MDCK II cells (Fig. S1A,B) and because re-expression of an shRNA-resistant form of Slp2-a (Slp2-a SR) in the Slp2-a-KD cells restored them to normal cell size (Fig. 1D). Because Slp2-a was originally described as a Rab27 effector (Fukuda, 2006; Kuroda and Fukuda, 2004), we investigated whether the Rab27 binding ability of Slp2-a is required for control of cell size by using a Rab27-binding-deficient Slp2-a(E11A/R32A) mutant (Kuroda and Fukuda, 2004). As shown in Fig. 1D, the Slp2-a mutant protein was localized at the plasma membrane, the
same as the wild-type protein, and was able to restore the size of Slp2-a-KD cells. Consistent with these findings, functional ablation of Rab27A either by expression of a constitutively active mutant (Q78L) or negative mutant (T23N) of Rab27A (Fig. S1D), or by knockdown of endogenous Rab27A expression (Fig. S1E,F) had no effect on the size of the control MDCK II cells. Furthermore, the difference between the level of Rab27A expression in the control cells and the Slp2-a-KD cells was not observed (Fig. S1C). Taken together, these results indicate that Slp2-a regulates renal epithelial cell size independently of Rab27.

**Slp2-a interacts with Rap1GAP2**

We focused our exploration of the mechanism of the Slp2-a-mediated control of cell size on Rap1, because Rap1 is known to play a central role in basic cellular events, e.g., adhesion and spreading (Bos et al., 2001), and because Rap1GAP2, a GTPase-activating protein (GAP) for Rap1 (Schultess et al., 2005), has been shown to interact with Slp1 (Neumüller et al., 2009), the closest homologue of Slp2-a (Fukuda et al., 2001). First, we performed co-immunoprecipitation assays to test for a possible interaction between Slp2-a and Rap1GAP2. As expected, both Slp1 and Slp2-a interacted with Rap1GAP2 (Fig. 2A), and the Rap1GAP2-binding site in Slp2-a was mapped to the C2B domain (Fig. 2B, bottom, lane 5). To investigate the subcellular localization of Slp2-a and Rap1GAP2, we co-expressed C-terminal Venus (a variant of yellow fluorescent protein)-tagged Rap1GAP2 and monomeric red fluorescent protein (mRFP)-tagged Slp2-a in control MDCK II cells. Consistent with the results of the co-immunoprecipitation assays described above, Slp2-a and Rap1GAP2 were mainly co-localized at the plasma membrane (Fig. 2C). When Rap1GAP2 alone was
expressed in control MDCK II cells, Rap1GAP2 was also localized at the plasma membrane, whereas it was dispersed in the cytoplasm of Slp2-a-KD cells (Fig. 2D), indicating that Slp2-a is required for the plasma membrane-localization of Rap1GAP2.

**Disruption of the Slp2-a–Rap1GAP2 interaction affects cell size**

Because Rap1GAP2 has been shown to interact with Slp1 through its C-terminal EVTKTT sequence (amino acids 537-542 of Rap1GAP2) (Neumüller et al., 2009), we investigated whether Slp2-a also interacts with this sequence. To this end, we generated an N-terminal truncated mutant of Rap1GAP2 (amino acids 518-687 of Rap1GAP2; designated as RGDN) and a deletion mutant of the EVTKTT sequence from the RGDN (RGDN-ΔEVTKTT) (Fig. 3A, left), and then performed co-immunoprecipitation assays with the C2B domain of Slp2-a. As shown in Fig. 3A (right), the RGDN construct, but not the RGDN-ΔEVTKTT mutant, interacted with the C2B domain of Slp2-a, indicating that the EVTKTT sequence is also required for the interaction between Slp2-a and Rap1GAP2. If the interaction between Slp2-a and Rap1GAP2 were actually involved in the control of cell size, disruption of the interaction by a dominant negative construct, e.g., the binding site alone, should affect cell size. As expected, overexpression of Venus-tagged RGDN in control MDCK II cells resulted in an increase in cell size, whereas overexpression of the Slp2-a-binding-deficient mutant (RGDN-ΔEVTKTT) did not (Fig. 3B). Similarly, GFP-tagged Slp2-a-C2B exhibited a dominant negative effect, i.e., its overexpression in control MDCK II cells resulted in an increase in cell size (Fig. 3C), whereas GFP-tagged Slp2-a-C2A, which lacks Rap1GAP2 binding ability (Fig. 2B), had no effect on cell size (Fig. 3C). Taken together, these results indicate that Slp2-a...
regulates renal epithelial cell size through interaction with Rap1GAP2.

**Targeting of Rap1GAP2 to the plasma membrane is required for the control of cell size**

Because Rap1GAP2 was dispersed in the cytoplasm of the Slp2-a-KD cells (Fig. 2D), we hypothesized that Rap1GAP2 functions at the plasma membrane during the control of cell size. To test our hypothesis, we generated a plasma membrane-targeting Rap1GAP2 construct by using a plasma membrane-targeting sequence (CAAX) of K-Ras (Fig. 3A, left) (Kobayashi and Fukuda, 2013). Unlike wild-type Rap1GAP2, expression of Rap1GAP2-CAAX in Slp2-a-KD cells restored the cells to normal cell size (Fig. 4A). To determine whether the GAP activity of Rap1GAP2 is actually involved in the control of cell size, we generated an additional construct, Rap1GAP2(N357A)-CAAX, in which the mutation has been shown to completely impair GAP activity (Schultess et al., 2005), and evaluated its effect on the size of Slp2-a-KD cells. As expected, the mutant construct had no effect on cell size, even though it was clearly localized at the plasma membrane (Fig. 4A). These results indicate that targeting of Rap1GAP2 to the plasma membrane and its subsequent activation is required for control of cell size.

*Drosophila* Btsz is the only homologue of mammalian Slp that contains C-terminal tandem C2 domains but lacks an SHD (Fukuda et al., 2001; Pilot et al., 2006). Because Btsz mutations have been shown to affect growth and cell size in *Drosophila* (Serano and Rubin, 2003), we also investigated whether Btsz could compensate for the function of Slp2-a in MDCK II cells. Co-immunoprecipitation assays were performed to test for a possible interaction between a short form of Btsz
that contains only the tandem C2 domains and Rap1GAP2, and, as expected, Btsz interacted with Rap1GAP2, the same as Slp2-a did (Fig. 4B). Next, we expressed Venus-tagged Btsz in control and Slp2-a-KD cells and evaluated its effect on cell size. It is noteworthy that Btsz was localized at the plasma membrane and that expression of Btsz in the Slp2-a-KD cells restored normal cell size. Intriguingly, however, Btsz did not affect the size of control MDCK II cells (Fig. 4C). These results suggested that the Rap1GAP2 recruiting function of Slp in the control of cell size may have been conserved across species from *Drosophila* to humans.

**Rap signaling is activated in Slp2-a-KD cells**

Because Rap1GAP has the ability to inactivate both Rap1 and Rap2 (Gloerich and Bos, 2011), we measured the level of GTP-Rap1/2 in Slp2-a-KD cells as described in the Materials and Methods and found that both Rap1 and Rap2 activities (i.e., their GTP levels) were increased in the Slp2-a-KD cells (Figs. 5A, S2A). Although both Rap1 and Rap2 modulate Rac1 signaling, they have been reported to regulate cell size by different mechanisms: active Rap1 has been reported to regulate cell size in HeLa cells by promoting cell spreading by localizing Rac1 guanine nucleotide exchange factors (GEFs) (Arthur et al., 2004), whereas active Rap2 has been reported to regulate cell size in intestinal epithelial cells by activating an upstream target of Rac1 (Gloerich et al., 2012) independently of Rap1. We demonstrated that Rac1 is also activated in Slp2-a-KD cells (Figs. 5A, S2E), and then investigated whether activated Rap1 and Rap2 induce renal cell spreading by different mechanisms. We generated constitutively active and negative mutants (G12V and S17N, respectively) of Rap1A and Rap2A and expressed them in control MDCK II cells (Figs. 5B, S2B). As
expected, both active mutants induced cell spreading, but neither negative mutant did.
Intriguingly, when active Rap1A and Rap2A were simultaneously expressed in control MDCK II cells, comparison with cells expressing each of the active mutants showed that their cell size had increased additively (Fig. 5B), suggesting that Rap1 and Rap2 regulate the size of renal epithelial cells through different pathways. We next investigated the possible involvement of Rac1 in cell size by using constitutively active and negative mutants of Rac1 (G12V and T17N, respectively) (Figs. 5C, S2C). Expression of the active mutant of Rac1, but not of its negative mutant, in the control MDCK II cells induced cell spreading, whereas all of the negative mutants of Rap1A, Rap2A, and Rac1 decreased the size of the Slp2-a-KD cells (Figs. 5D, S2D), indicating that the Slp2-a–Rap1GAP2 interaction regulates renal epithelial cell size by modulating Rap signaling.

**Ezrin is activated in Slp2-a-KD cells**

Because Rap2 has been reported to activate ezrin (Gloerich et al., 2012), one of the ERM family proteins that bind F-actin and are involved in the regulation of cell spreading (Bretscher et al., 2002; Ross et al., 2011), and because increased phosphorylation of ezrin (active form) was shown in confluent Slp2-a-KD cells in our previous study (Yasuda et al., 2012), we hypothesized that the increased Rap2 activity in subconfluent Slp2-a-KD cells also activates ezrin. The results of the immunoblot analysis showed a clear increase in ezrin phosphorylation in the subconfluent Slp2-a-KD cells in comparison with the control MDCK II cells (Fig. 6A). Because activated ezrin is known to initiate activation of Rac1 through its ability to bind and sequester the Rho GDP dissociation inhibitor (Rho-GDI), which interacts with Rac1 and
inhibits Rac1 activity (Hsu et al., 2010; Yang and Hinds, 2006), we further hypothesized that activated ezrin and Rho-GDI induce cell spreading. To test our hypothesis, we generated a constitutively active mutant of ezrin, a phosphomimetic form of ezrin(T567D), and a C-terminal truncated mutant of ezrin (ezrin-N) that possesses Rho-GDI binding ability and evaluated their effect on cell size in control MDCK II cells. The results showed that both mutants, but not the wild-type (WT) ezrin, increased the size of control MDCK II cells (Fig. 6B), suggesting that activated ezrin induces cell spreading through Rac1 activation. In an attempt to further support our hypothesis, we co-expressed a constitutively negative mutant of either Rap2A or Rac1 together with an active mutant of ezrin(T567D) in control MDCK II cells (Fig. 6C). As expected, Rac1(T17N) antagonized the effect of ezrin(T567D), i.e., it inhibited the cell spreading induced by ezrin(T567D), whereas Rap2A(S17N) had no effect on ezrin(T567D)-mediated cell spreading. Taken together, these results strongly indicate that activated Rap2A facilitates activation of Rac1 through phosphorylation of ezrin, which in turn leads to cell spreading.

Increased ceramide levels have been shown to inhibit ezrin phosphorylation by activating protein phosphatases (PP1 and/or PP2A) (Canals et al., 2010; Canals et al., 2012; Zeidan et al., 2008) and to induce cell growth arrest (Shayman et al., 1991). The ceramide level of renal epithelial cells has been reported to be increased by glucosylceramide (GlcCer) synthase inhibitor treatment (Natoli et al., 2010; Shayman et al., 1991). We therefore investigated the effect of a GlcCer synthase inhibitor, miglustat (drug for the treatment of Niemann-Pick C disease) (Venier and Igdoura, 2012), on the ezrin activity of Slp2-a-KD cells and on their cell size (Fig. 6D,E). It should be noted that miglustat had the effect of inactivating the ezrin activity and
reducing cell size in the Slp2-a-KD cells.

**Mislocalization of Slp2-a in the cytoplasm induces increased MDCK II cell size**

To investigate whether mislocalization of Slp2-a from the plasma membrane affects cell size, we overexpressed GFP-tagged Slp2-a in control MDCK II cells (Fig. 7A). When the level of GFP-Slp2-a expression was relatively low (Fig. 7A, top right, open circles), GFP-Slp2-a was predominantly localized at the plasma membrane (Fig. 7A, top left, open circles) and the size of the GFP-Slp2-a-expressing MDCK II cells was almost the same as the size of the control GFP-expressing cells (Fig. 7A, left two panels in the lower images). As the GFP-Slp2-a expression level increased (Fig. 7A, top right, closed circles), however, the overexpressed GFP-Slp2-a was often dispersed in the cytoplasm and induced cell spreading (Fig. 7A, top left, closed circles; and bottom, right two panels). Because the interaction between Slp2-a and Rap1GAP2 was actually involved in the control of cell size (Figures 2-4), mislocalization of Slp2-a was expected to affect the localization of Rap1GAP2. Just as expected, when Rap1GAP2 was co-expressed with Slp2-a in control MDCK II cells (Fig. 7B), the localization patterns of Rap1GAP2 were similar to those of Slp2-a: cytoplasmic localization of Rap1GAP2 was often observed in MDCK II cells that expressed a high level of Slp2-a (Fig. 7B, left graphs, closed circles), as opposed to the plasma membrane-localization of Rap1GAP2 in MDCK II cells that expressed Rap1GAP2 alone (Fig. 7B, lower left graphs, red triangles). Because, as noted above, plasma membrane-localization of Slp2-a and Rap1GAP2 are required for proper cell signaling, mislocalization of Slp2-a and Rap1GAP2 in the cytoplasm as a result of Slp2-a overexpression may lead to abnormal signaling in the control of cell size.
Increased Slp2-a expression and ezrin activation in the renal cyst epithelium of the kidney of pcy mice

Because Slp2-a regulates the size of MDCK II cells (a renal epithelial cell line) (Fig. 1) and is expressed in mouse renal proximal tubules (Fig. 8B, top two rows) (Yasuda et al., 2012), in the final set of experiments we investigated the possibility of a link between dysfunction of Slp2-a-mediated signaling and cell spreading-related disorders in the kidney (Hildebrandt et al., 2009; Torres and Harris, 2009; Wilson, 2004). Pcy mice, a spontaneous model of late-onset (adolescent) type of human NPHP that is characterized by multicystic dysplastic kidneys, situs inversus, and congenital heart defects (Bergmann et al., 2008; Olbrich et al., 2003), have very large kidneys (Fig. 8A). Intriguingly, Slp2-a was found to be expressed in the renal cyst epithelium of pcy mice (Fig. 8B, arrowheads in the bottom panel) in addition to the renal proximal tubules, where Slp2-a is normally expressed in the WT mice. When we then compared the Slp2-a expression level in the kidneys of WT mice and pcy mice (Fig. 8C), we found that the Slp2-a expression level was clearly higher in the kidneys of the pcy mice, but that the expression levels of other Slp family proteins were not. A reverse transcriptase (RT)-PCR analysis showed that Slp2-a expression in pcy mice was also increased at the mRNA expression level, but that expression of Slp1, Rap1GAP1, and Rap1GAP2 mRNAs was unaltered (Fig. 8D). Because a proper amount of Slp2-a is required for the control of MDCK II cell size (i.e., both knockdown and overexpression of Slp2-a induce cell spreading) (Figs. 1C, 7A), it is highly possible that the increased amount of Slp2-a in pcy mice causes abnormal signaling in their renal cyst epithelium. To investigate this possibility, we searched for the cell signaling responsible for cyst
expansion in the kidneys of \textit{pcy} mice (Fig. 8E). As previously reported (Omori et al., 2006; Torres and Harris, 2006), the mitogen-activated protein kinase (MAPK) pathway was activated because of the increased expression of epidermal growth factor receptor (EGFR) in the kidneys of \textit{pcy} mice (Fig. 8E, upper three panels in the left blots), and the cAMP-dependent protein kinase A (PKA)–CREB pathway was activated by the increased level of circulating vasopressin in the kidneys of \textit{pcy} mice (Gattone et al., 2003; Torres and Harris, 2009) (Fig. 8E, lower two panels in the left blots). Intriguingly, we found that phosphorylation of ezrin was clearly increased in the kidneys of \textit{pcy} mice, and that the ezrin–phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Gautreau et al., 1999) was activated as a result (Fig. 8E, right blots), suggesting that ezrin signaling is involved in the control of cell signaling in the kidneys of \textit{pcy} mice. Because ezrin phosphorylation was increased in the kidneys of \textit{pcy} mice, we proceeded to compare the localization of activated ezrin and Slp2-a in the kidneys of WT and \textit{pcy} mice (Fig. 8F,G). The results showed that most of the ezrin in both the WT and \textit{pcy} mice was activated and localized at the brush border (apical membrane) of the epithelial cells of the renal proximal tubules (Bretscher et al., 2002; Hatano et al., 2013), where Slp2-a was abundantly expressed (Yasuda et al., 2012). In the \textit{pcy} mice, however, activated ezrin was also found to be localized at the basolateral membrane of the renal cyst epithelium (Fig. 8F,G, arrowheads in the bottom panels), where Slp2-a was also expressed. Because we and others had previously shown that excessively activated ezrin was mislocalized to the basolateral membrane (Pujuguet et al., 2003; Yasuda et al., 2012), this finding suggested that an excess amount of Slp2-a leads to excessive activation of ezrin in renal cyst epithelial cells, and consequently to cell spreading.
Discussion

The Slp family consists of five members (Slp1-5) in humans and mice (Fukuda et al., 2001; Fukuda and Mikoshiba, 2001; Kuroda et al., 2002), and all members share an N-terminal SHD and C-terminal tandem C2 domains. The SHD of Slps specifically interacts with an active form of Rab27 (Kuroda et al., 2002), whereas the C2 domains, especially the C2A domain, interact with phospholipids in the plasma membrane (Catz et al., 2001; Fukuda, 2002; Kuroda and Fukuda, 2004; Gálvez-Santisteban et al., 2012). All of the Slp family members have been reported to regulate the movement of Rab27-bearing vesicles/organelles and/or their docking to the plasma membrane via the N-terminal SHD (Fukuda, 2006), but whether they have additional functions other than the Rab27 effector function had never been determined. In the present study we discovered a novel function of Slp2-a in the control of renal epithelial cell size independently of Rab27 (Figs. 1, S1). Slp2-a, but not Slp1 (Fig. S1C,G,H), controls renal epithelial cell size by recruiting Rap1GAP2 to the plasma membrane through its interaction with the C2B domain of Slp2-a (Figs. 2, 3), and the Rap1GAP2 recruiting function (i.e., Slp-mediated Rap signaling) appears to have been conserved across species from Drosophila to humans (Fig. 4B,C).

Rap1 and Rap2 coordinately regulate cell spreading through Rac1 activation and through ezrin activation, respectively (Figs. 5, 6). Rap signaling is controlled by specific guanine nucleotide exchange factors (GEFs) and GAPs (Gloerich and Bos, 2011), and we have succeeded in showing a functional link between Rap signaling and Slp2-a. We found that Slp2-a interacts with both Rap1GAP1 (Fig. S3A) and Rap1GAP2 (Fig. 2A), both of which are expressed in mouse kidneys (Fig. 8D), and that
it anchors them to the plasma membrane (Fig. 2C,D). Dysregulation of Slp2-a either by knockdown or by overexpression (i.e., mislocalization of Slp2-a and Rap1GAP2 in the cytoplasm) induced cell spreading in renal epithelial cells (Figs. 1C, 7, S1B), indicating that the activity of Rap1 and Rap2 is regulated in close proximity to the plasma membrane by Rap1GAPs. Because activation of Rap signaling, Rac signaling, and/or ezrin signaling, has been shown to impair cell-cell adhesion by destabilizing E-cadherin at the cell-cell junctions (Lozano et al., 2008; Pujuguet et al., 2003; Tsygankova et al., 2010) and because reduced accumulation of E-cadherin at the cell-cell junctions was observed in the Slp2-a-KD cells (Fig. S4A), it is highly possible that activation of these signalings facilitates cell spreading by disrupting cell-cell adhesion.

We also present evidence for the link between impairment of Slp2-a-mediated Rap–ezrin signaling and PKD. PKD is the most common inherited renal cystic disease and a leading cause of end-stage renal disease. They occur worldwide and in all races with a prevalence estimated to be between 1:400 and 1:1000. Dysfunctions of several cell signaling pathways, including the excess activation of EGFR–MAPK pathway and excess activation of cAMP–PKA pathway due to an increased circulating vasopressin level, have been reported to contribute to the pathogenesis of PKD (Torres and Harris, 2006; Torres and Harris, 2009) (Fig. 8E). In addition to these activated signaling pathways, we found that Slp2-a expression is increased in the kidneys of pcy mice (Fig. 8C). Because a candidate CREB-binding site was identified in the mouse Slp2-a gene by a search of the CREB Target Gene Database (http://natural.salk.edu/CREB/), it is highly possible that Slp2-a mRNA transcription was activated by CREB through PKA phosphorylation. Actually, Slp2-a expression in the control MDCK II cells was
dose-dependently increased in response to stimulation with a cAMP-elevating reagent, forskolin (Fig. S3B), and their cell size increased in tandem (Fig. S3C). cAMP also activates the cAMP-dependent RapGEFs (cAMP-GEFs) that activate both Rap1 and Rap2, and cAMP-GEFs have been shown to be expressed in the kidney (Li et al., 2008). Since activation of cAMP-GEFs induced cell spreading in renal epithelial cells (Fig. S3D) (Ross et al., 2011), Rap signaling must also be enhanced and contribute to the pathogenesis of PKD. Because membrane polarity proteins, e.g., E-cadherin, are known to be destabilized in the kidneys of PKD and NPHP patients (Charron et al., 2000; Marciano et al., 2011; Okada et al., 2000; Rocco et al., 1992; Wilson, 2011), and because we also observed a similar phenotype in pcy mice (Fig. S4B), it is likely that both impairment of Slp2-a-mediated Rap–ezrin signaling and enhancement of cAMP-induced RapGEF signaling ultimately activate Rac1 and that the activated Rac1 causes cell spreading by destabilizing E-cadherin at the cell-cell junctions in the kidneys of pcy mice (Fig. S5, right).

Preclinical studies of several agents that decrease the intracellular cAMP level have shown that they reduce cell growth in the cystic kidneys of PKD patients (Gattone et al., 2003; Torres and Harris, 2009; Torres et al., 2012). However, because the decrease in intracellular cAMP level has the undesirable effect of decreasing expression of the water channel aquaporin-2 and its apical membrane-localization (Nielsen et al., 2002), the aquaretic side effects (e.g., thirst, polyurea, nocturia, and polydipsia) of treatment with such agents in PKD patients are an important issue that needs to be resolved. Natoli et al. recently reported that GlcCer and ganglioside GM3 levels were increased in human and mouse PKD kidneys and that blockade of GlcCer accumulation with a GlcCer synthase inhibitor effectively inhibited cystogenesis in mouse models of
PKD (PKD1 conditional knockout mice) and NPHP (jck and pcy mice) by largely unknown mechanisms (Natoli et al., 2010). It should be noted that the ceramide level of renal epithelial cells is increased by GlcCer synthase inhibitor treatment (Natoli et al., 2010; Shayman et al., 1991) and that increased ceramide levels have been shown to inhibit ezrin phosphorylation by activating protein phosphatases (PP1 and/or PP2A) (Canals et al., 2010; Canals et al., 2012; Zeidan et al., 2008) and to induce cell growth arrest (Shayman et al., 1991). We also found that the GlcCer synthase inhibitor miglustat has the effect of inactivating the ezrin–PI3K/AKT pathway and of reducing the size of Slp2-a-KD cells (Fig. 6D,E). Taken together, these findings indicate that inactivating Rac1 as a means of slowing cell growth in renal epithelial cells (Figs. 5D, 6C) should be an effective approach to curing PKD patients, because ezrin regulates Rac1 activity through Rho-GDI (Hsu et al., 2010; Yang and Hinds, 2006) (Fig. S5, right). Thus, ezrin (or glycosphingolipid metabolism) should be a promising therapeutic target for PKD, and it will be necessary to search for new drugs that antagonize ezrin signaling to treat PKD patients.

In summary, we have identified Rap1GAPs as novel interactors for the Slp2-a-C2B domain and discovered that their interactions are crucial for the control of renal epithelial cell size independently of the known Rab27 effector function of Slp2-a. In the absence of the proper level of Slp2-a-mediated signaling, renal epithelial cells exhibit a cell spreading phenotype due to increased Rap–ezrin signaling, and the increased Rap–ezrin signaling is likely to be associated with the renal cell spreading that occurs in PKD. Hence, the Slp2-a-mediated Rap–ezrin signaling that controls renal epithelial cell size may provide a novel target for the treatment of PKD.
Materials and Methods

Materials

Horseradish peroxidase (HRP)-conjugated anti-T7 tag mouse monoclonal antibody and anti-T7 tag mouse monoclonal antibody-conjugated agarose were purchased from Merck Biosciences Novagen (Darmstadt, Germany). HRP-conjugated anti-GAPDH mouse monoclonal antibody, HRP-conjugated anti-GFP rabbit polyclonal antibody, and HRP-conjugated anti-red fluorescent protein rabbit polyclonal antibody were obtained from MBL (Nagoya, Japan). HRP-conjugated anti-FLAG tag (M2) mouse monoclonal antibody and anti-FLAG tag mouse monoclonal antibody-conjugated agarose were from Sigma-Aldrich (St. Louis, MO). HRP-conjugated anti-GST rabbit polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ezrin mouse monoclonal antibody, anti-CREB rabbit polyclonal antibody, and anti-CREB (phospho S133) rabbit polyclonal antibody were from Abcam (Cambridge, MA). Anti-AKT mouse monoclonal antibody and anti-E-cadherin mouse monoclonal antibody were from BD Transduction Laboratories (Lexington, KY). Anti-phospho-ezrin(Thr567)/radixin(Thr564)/moesin(Thr558) (anti-phospho-ERM) rabbit polyclonal antibody, anti-p44/42 MAPK (ERK1/2) rabbit polyclonal antibody, anti-phospho-p44/42 (ERK1/2) (Thr202/Tyr204) mouse monoclonal antibody, and anti-phospho-AKT (Thr308) rabbit polyclonal antibody were from Cell Signaling Technology (Danvers, MA). Anti-claudin-2 rabbit polyclonal antibody and the Alexa-Fluor-488/594-conjugated goat secondary antibodies were from Invitrogen (Carlsbad, CA). Anti-EGFR sheep polyclonal antibody was from Fitzgerald (Concord, MA). Anti-Slp1, anti-Slp2-a, anti-Slp3-a, anti-Slp4-a, anti-Slp5, and anti-Rab27A
rabbit polyclonal antibodies were prepared as described previously (Imai et al., 2004). Three stealth RNAi™ siRNAs against canine Slp2-a site 1 (target site #1: 5’-GAAGAAGCTTCAGGTAGCAGCTGAA-3’), site 2 (target site #2: 5’-GAGACTTTTGGAATCTGGAAGTAAA-3’), and site 3 (target site #3: 5’-CCCTGCTCTCAGAATGCTGTTGAT-3’) were synthesized by Invitrogen. Three siRNAs against canine/human Rab27A site 1 (19-base target site #1: 5’-CCAGTGTTACTTTACCAATA-3’) and site 2 (19-base target site #2: 5’-GGAGAGGTTTCGTAGCTTA-3’) and site 3 (19-base target site #3: 5’-CGACAGCGTCTTTCCAGAGA-3’) were synthesized by Nippon Gene Material Co., Ltd. (Toyama, Japan). DBA/2Jc/e mouse kidneys and pcy mouse kidneys were purchased from CLEA Japan Inc. (Tokyo, Japan) and KYUDO Co. (Saga, Japan), respectively.

**Plasmid construction**

pEGFP-C1 (Clontech-Takara Bio, Shiga, Japan), pmRFP-C1, and/or pEF-T7 tag expression vectors harboring cDNAs of mouse Slp2-a and Rab27A and their mutants were prepared essentially as described previously (Fukuda and Kuroda, 2002; Kuroda and Fukuda, 2004). cDNAs encoding human Rap1A, Rap2A, Rap1GAP1, and Rap1GAP2, and mouse ezrin were amplified from Marathon-Ready adult human or mouse brain and testis cDNA (Clontech-Takara Bio) and *Drosophila* Btsz was amplified from a *Drosophila* cDNA library (Merck Biosciences Novagen) by PCR with the following pairs of oligonucleotides having a restriction enzyme site (underlined) as described previously (Fukuda et al., 1999): 5’-GGATCCATGCGGTGAGTACACAGCTAGT-3’ and
5’-GTCGACCTAGAGCAGCAGACATGATT-3’ for Rap1A, 
5’-GGATCCATGCGCGAGTACAAAGTGGT-3’ and 
5’-GTCGACCTATTGTATGTTACATGCAG-3’ for Rap2A, 
5’-GGATCCATGAGCGGCCGGAAGCGCA-3’ and 
5’-CTAACAGCCCAGCTGGGGCAT-3’ for Rap1GAP1, 
5’-AGATCTATGTTTGGCCGGAAGCGCAG-3’ and 
5’-GTCGACTTAGTGACCCGCACCAGAGC-3’ for Rap1GAP2, 
5’-GGATCCCTCGACAGTGAGGGGCATCCCTTGCC-3’ and 
5’-GTCGACCTAGCTGGGGCTCTCGCTGCTCGG-3’ for RGDN, 
5’-AGATCTCCACCATGCCCCAGCCAATCAACG-3’ and 
5’-GGTACCCGGGCCTGGGCCTTCATCTGCTG-3’ for the N-terminal domain of ezrin, 
5’-AGATCTGGCCACCAGTGAGGGGCCAGGCCCCGG-3’ and 
5’-GGTACCCATGCGCGAGTACAAAGTGGT-3’ for Rac1(G12V), Rac1(T17N), 
5’-AGATCTGGCCACCAGTGAGGGGCCAGGCCCCGG-3’ and Rac1(G12V), Rac1(T17N), ezrin(T567D), Rap1GAP2(N357A), and RGDN-ΔEVTKTT 
were produced by conventional or two-step PCR techniques as described previously (Tamura et al., 2011). shRNA targeted to canine Slp1 (21-base target site: 
5’-GCGCAAGACCACAGTGAAAGA-3’) was constructed as described previously 
(Kuroda and Fukuda, 2004), by using the pSilencer-neo 2.0-U6 vector (Ambion, Austin,
TX), which expresses shRNA.

**Cell culture, transfection, and drug treatment**

MDCK II cells and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 µg/ml). Plasmids were transfected into MDCK II cells or COS-7 cells by using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. MDCK II cells were cultured in serum-free DMEM for 16 hours and then treated with 100 nM, 500 nM, or 1 µM forskolin (Wako Pure Chemical Industries) for 5 minutes (to detect CREB activity) or for 48 hours (to detect Slp2-a expression). Slp2-a-KD cells were also treated with 10 µM or 50 µM miglustat (Sigma-Aldrich) for 24 hours.

**Establishment of Slp2-a-knockdown cell lines by RNA interference**

Control MDCK II cells and stable Slp2-a-knockdown MDCK II cells were established as described previously (cell line #2 in Yasuda et al., 2012). The cells were grown in DMEM containing 800 µg/ml G418 (Invitrogen). Stealth siRNAs against canine Slp2-a were transfected into MDCK II cells by using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions.

**Total RNA preparation and RT-PCR analysis**

Total RNA preparation and the RT-PCR analyses were performed as described previously (Yasuda et al., 2012). The mouse Slp1, Slp2-a, Rap1GAP1, and Rap1GAP2 primer pairs were designed to amplify fragments of, 532 bp, 447 bp, 715 bp,
and 511 bp, respectively. The primers were used for Slp1 (forward primer, 5’-GCAGATCTTCTCCAGATGAGCTTCCCAG-3’ and reverse primer, 5’-GCGAATTCTGCCCTGGGGACCAGGTT-3’), Slp2-a (forward primer, 5’-GTTGCCCTCGAGACAGAAAAC-3’ and reverse primer, 5’-CATCTTTCTCCCAGAGAGCAA-3’), Rap1GAP1 (forward primer, 5’-ATCAGAAGCTTGGGCAGACCT-3’ and reverse primer, 5’-CTCCGGATGACCCGCTTGAAA-3’), and Rap1GAP2 (forward primer, 5’-AGATGCTGGAGAAAATGCAG-3’ and reverse primer, 5’-GCATCATCACAGAAAGCCTT-3’). The mouse G3PDH (glyceraldehyde 3-phosphate dehydrogenase; GAPDH) was amplified using mouse G3PDH-5’ and G3PDH-3’ primers (BD Biosciences Clontech) as a reference. cDNAs were amplified by PCR using rTaq DNA polymerase (Toyobo) with 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1.5 min.

**Immunofluorescence analysis**

Stable and transfected MDCK II cells were cultured on glass-bottomed dishes (35-mm dish; MatTek, Ashland, MA) for 24 hours and then fixed with 4% paraformaldehyde for 10 minutes at room temperature. To visualize cell-cell adhesion, cells were permeabilized with 0.3% Triton X-100 in PBS for 2 minutes and blocked with the blocking buffer (1% BSA and 0.1% Triton X-100 in PBS) for 1 hour at room temperature. The cells were then incubated for 2 hours at room temperature in the same buffer containing anti-E-cadherin antibody (1/100 dilution) followed by incubation with secondary antibodies (Alexa 488-conjugated antibodies; Invitrogen) and stained with DAPI. To measure cell size, cells were stained with Texas
Red-conjugated phalloidin (1/200 dilution; Invitrogen). The stained cells were examined for immunofluorescence signals with a confocal fluorescence microscope (Fluoview 1000; Olympus, Tokyo, Japan) and with MetaMorph software (Molecular Devices, Sunnyvale, CA) (for Fig. 2C,D). The immunofluorescence data shown in this paper are single plane images of cells. The immunofluorescence intensity was quantified with ImageJ software (version 1.44o; NIH) (for Figs. 7, S4A).

**Immunoblot analysis**

MDCK II cells (1 × 10^5 cells/6-cm dish) were cultured for 48 hours and harvested. After washing the cells with PBS and lysing them in a lysis buffer (50 mM HEPES-KOH, pH7.2, 150 mM NaCl, 1 mM MgCl₂, 1% Triton X-100, a protease inhibitor cocktail (Roche, Mannheim, Germany), and phosphatase inhibitor cocktail (Sigma-Aldrich)), the cell lysates were subjected to 10% SDS-PAGE followed by immunoblotting with specific primary antibodies indicated in each figure. Immunoreactive bands were visualized with appropriate HRP-conjugated secondary antibodies (GE Healthcare Ltd., Little Chalfont, UK) and detected by enhanced chemiluminescence (ECL, GE Healthcare Ltd.). The blots shown in this paper are representative of three or four independent experiments. Mouse kidneys were lysed in the same lysis buffer and analyzed by 10% SDS-PAGE as described above. The intensity of the immunoreactive bands was quantified with ImageJ software.

**Co-immunoprecipitation assays**

COS-7 cells (3 × 10^5 cells/6-cm dish, the day before transfection) were transfected with plasmids (pEF-T7-Slps and pEF-FLAG-Rap1GAPs), cultured for 2 days, and harvested.
Co-immunoprecipitation assays with anti-FLAG tag or anti-T7 tag antibody-conjugated agarose were performed as described previously (Kuroda et al., 2002).

**Assays for Rap activity and Rac activity**

MDCK II cells were cultured for 48 hours and lysed in lysis buffer to assay for Rap activity (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM MgCl₂, 1% NP-40, 1 mM DTT, 10% glycerol, protease inhibitor cocktail, and phosphatase inhibitor cocktail), or in lysis buffer to assay for Rac activity (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 500 µM DTT, 15% glycerol, protease inhibitor cocktail, and phosphatase inhibitor cocktail). The cell lysates were centrifuged at 15,000 ×g for 10 minutes, and 1 mg samples were incubated at 4°C for 2 hours with glutathione-Sepharose 4B beads (GE Healthcare Ltd.) coupled with 30 µg of GST alone as a negative control, GST-RalGDS (for Rap), or GST-PAK1-PBD (for Rac). After washing the beads three times with the lysis buffer, proteins trapped by the beads (active Rap or active Rac1) and total lysates (total Rap or total Rac1) were analyzed by immunoblotting with anti-Rap antibody or anti-Rac1 antibody.

**Immunohistochemical analysis**

Immunohistochemical staining was performed as described previously (Yasuda et al., 2012). The samples were stained with anti-E-cadherin antibody, anti-Slp2-a antibody, anti-claudin-2 antibody, anti-ezrin antibody, and anti-P-ERM antibody. To double stain for Slp2-a and claudin-2 or for Slp2-a and P-ERM, we directly labeled Slp2-a antibody with Alexa Fluor 488 or 594 by using a direct labeling kit (Zenon Alexa Fluor 488 or 594 rabbit IgG labeling kit; Invitrogen). The immunostained sections were
examined with a confocal fluorescence microscope.

**Statistical analyses**

The data are expressed as means and s.e.m. Data were tested for statistically significant differences by using Student’s unpaired *t*-test, Dunnet’s method, or the Tukey-Kramer method as indicated in the legend of each figure. A probability level of *p* < 0.05 was considered statistically significant.
Acknowledgments

We thank Megumi Aizawa for technical assistance and members of the Fukuda Laboratory for valuable discussions.

Author contributions

T.Y. designed and performed the experiments, and wrote the manuscript. M.F. supervised the project and wrote the manuscript.

Funding

This work was supported in part by JSPS KAKENHI Grant Number 242766 (to T.Y.) and by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology of Japan [grant numbers 24370077 and 24657125 to M.F.].

Supplementary material is available at Journal of Cell Science online.
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Figure legends

Figure 1: Loss of Slp2-a leads to increased the size of MDCK II cells. (A) Slp2-a expression in control and Slp2-a-KD cells. Note that the level of Slp2-a protein expression in the Slp2-a-KD cells was approximately only 5% of the level in the control cells. GAPDH was used as an internal control. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (B) Growth curve of control and Slp2-a-KD cells. 50,000 cells were seeded in individual 10-cm dishes at day 0, and trypsinized cells were counted at the times indicated. (C) Increased size of Slp2-a-KD cells. (Left) Control and Slp2-a-KD cells were cultured for 24 hours, fixed, permeabilized, and stained with Texas Red-conjugated phalloidin (for actin staining; red). The size of each cell was determined by measuring the area that stained positive for actin. Nuclei were stained with DAPI (blue). Scale bars, 10 μm. (Right) Quantification of the cell sizes shown in the images on the left. *p <0.001, Student’s unpaired t-test (n = 50 from three independent experiments). (D) Rescue experiments in Slp2-a-KD cells. (Left) Slp2-a-KD cells were transfected with pEGFP-C1, pEGFP-C1-Slp2-a SR, or pEGFP-C1-Slp2-a SR(E11A/R32A) (green), and cultured for 48 hours. The cells were re-plated on 35-mm glass-bottomed dishes, cultured for 24 hours, and then stained with Texas Red-conjugated phalloidin (red). Nuclei were stained with DAPI (blue). Scale bars, 10 μm. (Right) Quantification of the cell sizes shown in the images on the left. *p <0.001, Dunnett’s method (n = 55 from three independent experiments). The bars represent means and s.e.m.

Figure 2: Slp2-a is required for the plasma membrane-localization of Rap1GAP2. (A)
Interaction between Slp1/2-a and Rap1GAP2. T7-tagged Slp1 (or Slp2-a) and FLAG-tagged Rap1GAP2 were co-expressed in COS-7 cells, and their associations were analyzed by co-immunoprecipitation assays with anti-FLAG tag antibody-conjugated agarose beads. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (B) Mapping of the Rap1GAP2-binding site in Slp2-a. (Top) Schematic representation of the domain organization of Slp2-a. The truncated mutants of Slp2-a used in the co-immunoprecipitation assays are represented by solid lines. The Rap1GAP2 binding activity of each mutant is shown on the right. SHD, Slp homology domain; C2A/C2B, C2A/C2B domain. (Bottom) T7-tagged Slp2-a truncated mutants and FLAG-tagged Rap1GAP2 were co-expressed in COS-7 cells, and their associations were analyzed as described in A. The asterisks indicate immunoglobulin G heavy and light chains. (C) Co-localization of Slp2-a and Rap1GAP2. MDCK II cells were co-transfected with pVenus-N1-Rap1GAP2 (green) and pmRFP-C1-Slp2-a (red) and cultured for 48 hours. The cells were re-plated on 35-mm glass-bottomed dishes for 4 hours and then fixed. Nuclei were stained with DAPI (blue). Fluorescence intensity along the broken white lines (left panels) is shown on the right. The colocalization rate of Rap1GAP2 with Slp2-a was 59.2 ± 4% (n = 25). (D) Plasma membrane-localization of Rap1GAP2. Control and Slp2-a-KD cells were transfected with pVenus-N1-Rap1GAP2 (green) and cultured for 48 hours. The cells were re-plated on 35-mm glass-bottomed dishes, cultured for 4 hours, fixed, permeabilized, and stained with Texas Red-conjugated phalloidin (red). Nuclei were stained with DAPI (blue). Fluorescence intensity along the broken white lines (upper panels) is shown at the bottom. Scale bars, 10 μm.
**Figure 3**: Functional disruption of the Slp2-a–Rap1GAP2 interaction leads to cell spreading. (A) The Slp2-a-binding site in Rap1GAP2. (Left) Schematic representation of the functional domains of Rap1GAP2 and its deletion mutants and point mutants used in this study. The Slp2-a-C2B binding activity of each mutant is shown on the right. The CAAX after the C-terminus of Rap1GAP2 represents the plasma membrane-targeting sequence, and the A within the GAP domain represents an N357A substitution, which impairs GAP activity. (Right) T7-tagged Slp2-a-C2B and FLAG-tagged RGDN or RGDN-ΔEVTKTT were co-expressed in COS-7 cells, and their associations were analyzed by co-immunoprecipitation assays with anti-T7 tag antibody-conjugated agarose beads. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (B) Effect of the RGDN of Rap1GAP2 on cell size. (Left) MDCK II cells were transfected with pVenus-C1, pVenus-C1-RGDN, or pVenus-C1-RGDN-ΔEVTKTT (green), and cultured for 48 hours. The cells were re-plated on 35-mm glass-bottomed dishes, cultured for 24 hours, and then stained with Texas Red-conjugated phalloidin (red). The size of each cell was determined by measuring the area that stained positive for actin. Note that both the RGDN construct and RGDN-ΔEVTKTT construct were dispersed in the cytoplasm. Nuclei were stained with DAPI (blue). Scale bars, 10 µm. (Right) Quantification of the cell sizes shown in the images on the left. *p < 0.001, Dunnett’s method (n = 50 from three independent experiments). (C) Effect of the C2B domain of Slp2-a on cell size. (Left) MDCK II cells were transfected with pEGFP-C1, pEGFP-C1-Slp2-a-C2A, or pEGFP-C1-Slp2-a-C2B (green), and their cell size was measured as described above. Note that GFP-Slp2-a-C2A was localized at the plasma membrane, whereas GFP-Slp2-a-C2B was mainly present in the cytoplasm. Scale bars, 10 µm. (Right)
Quantification of the cell sizes shown in the images on the left. *p < 0.001, Dunnett’s method (n = 45 from three independent experiments). The bars represent means and s.e.m.

**Figure 4:** Plasma membrane-localization of Rap1GAP2 is required for the control of cell size. (A) Effect of forcible targeting of Rap1GAP2 to the plasma membrane on the size of Slp2-a-KD cells. (Left) Slp2-a-KD cells were transfected with pEGFP-C1, pVenus-N1-Rap1GAP2, pEGFP-C1-Rap1GAP2-CAAX, or pEGFP-C1-Rap1GAP2(N357A)-CAAX (green), and their cell size was measured as described in the legend of Fig. 1. In contrast to the cytoplasmic localization of Rap1GAP2, both Rap1GAP2-CAAX and Rap1GAP2(N357A)-CAAX (see also Fig. 3A) were localized at the plasma membrane in the Slp2-a-KD cells. Scale bars, 10 µm. (Right) Quantification of the cell sizes shown in the images on the left. *p < 0.001, Tukey-Kramer method (n = 40 from three independent experiments). (B) Interaction between Btsz and Rap1GAP2. T7-tagged Btsz (or Slp2-a) and FLAG-tagged Rap1GAP2 were co-expressed in COS-7 cells, and their associations were analyzed by co-immunoprecipitation assays with anti-FLAG tag antibody-conjugated agarose beads. The asterisks indicate immunoglobulin G heavy chain. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (C) Effect of the Btsz on cell size. (Left) Control cells and Slp2-a-KD cells were transfected with pVenus-C1 or pVenus-C1-Btsz (green). Btsz was localized at the plasma membrane in both control and Slp2-a-KD cells. Scale bars, 10 µm. (Right) Quantification of the cell sizes shown in the images on the left. *p < 0.001, Student’s unpaired t-test (n = 45 from three independent experiments). The bars represent means and s.e.m.
Figure 5: Rap signaling underlying the control of cell size in renal epithelial cells. (A) Activation of Rap signaling in Slp2-a-KD cells. Active Rap1, Rap2, and Rac1 were analyzed by pulldown assays and immunoblotting as described in the Materials and Methods. The results of negative control experiments are shown in Fig. S2A. GAPDH was used as an internal control. The top and middle panels indicate active and total small G proteins, respectively. The bottom panels indicate the GST fusion proteins that were used for the pulldown assays. Rap1, Rap2, and Rac1 were activated in the Slp2-a-KD cells. The amounts of active Rap1, Rap2, and Rac1 in the Slp2-a-KD cells expressed as a percentage of their amounts in the control cells were 217 ± 16% (n=3), 326 ± 51% (n=3), and 160 ± 20% (n=5), respectively, of the control cells. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (B) Modulation of cell size by manipulating Rap signaling. (Left) MDCK II cells were transfected with pVenus-C1, pVenus-C1-Rap1A(G12V), pVenus-C1-Rap1A(S17N), pVenus-C1-Rap2A(G12V), or pVenus-C1-Rap2A(S17N) (green), and co-transfected with pVenus-C1-Rap1A(G12V) (green) and pmStr-C1-Rap2A(G12V) (red), and their cell size was measured as described in the legend of Fig. 1. The expression levels of the fluorescent proteins used here are shown in Fig. S2B-D. Scale bars, 10 μm. (Right) Quantification of the cell sizes shown in the images on the left. *p <0.001, Tukey-Kramer method (n = 46 from three independent experiments). (C) Modulation of cell size by manipulating Rac1. (Left) MDCK II cells were transfected with pEGFP-C1, pEGFP-C1-Rac1(G12V), or pEGFP-C1-Rac1(T17N) (green). Scale bars, 10 μm. (Right) Quantification of the cell sizes shown in the images on the left. *p <0.001, Dunnett’s method (n = 70 from three independent experiments). (D) Effect of
inhibition of Rap signaling on the size of Slp2-a-KD cells. (Left) Slp2-a-KD cells were transfected with pEGFP-C1, pVenus-C1-Rap1A(S17N), pVenus-C1-Rap2A(S17N) or pEGFP-C1-Rac1(T17N) (green). Scale bars, 10 μm. (Right) Quantification of the cell sizes shown in the images on the left. *p <0.001 Dunnett’s method (n = 50 from three independent experiments).

Figure 6: Ezrin signaling underlying the control of cell size in renal epithelial cells. (A) Activation of ezrin in Slp2-a-KD cells. The cell lysates were analyzed by immunoblotting with the antibodies indicated. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (B) Modulation of cell size by manipulating ezrin. (Left) MDCK II cells were transfected with pVenus-C1, pVenus-N1-Ezrin(WT), pVenus-N1-Ezrin(T567D), or pEGFP-N1-Ezrin-N, and their cell size was measured as described in the legend of Fig. 1. Scale bars, 10 μm. (Right) Quantification of the cell sizes shown in the images on the left. *p <0.001, Dunnett’s method (n = 30 from three independent experiments). (C) Activation of ezrin by Rap signaling. (Left) MDCK II cells were transfected with pEGFP-C1, pVenus-C1-Rap2A(S17N), or pEGFP-C1-Rac1(T17N) together with pmStr-N1-Ezrin(T567D) (or pEGFP-C1 together with pmStr-N1). (Right) Quantification of the cell sizes shown in the images on the left. *p <0.001 Dunnett’s method (n = 30 from three independent experiments). (D) Inhibition of ezrin activity by miglustat. Slp2-a-KD cells were treated with miglustat (10 μM or 50 μM) for 24 hours and harvested. Cell lysates were analyzed by immunoblotting with the antibodies indicated. (E) Decreased size of Slp2-a-KD cells in response to miglustat. Slp2-a-KD cells were treated with miglustat (10 μM or 50 μM) for 48 hours and then
re-plated on the 35-mm glass-bottomed dishes and cultured with miglustat (10 μM or 50 μM) for 24 hours. The cells were stained with Texas Red-conjugated phalloidin to measure cell size. Quantification of their cell size is shown. *p <0.001, Dunnett’s method (n = 70 from three independent experiments). The bars represent means and s.e.m.

**Figure 7**: Mislocalizations of Slp2-a and Rap1GAP2 in the cytoplasm lead to cell spreading. (A) Overexpression of Slp2-a in MDCK II cells. (Bottom) MDCK II cells were transfected with pEGFP-C1 or pEGFP-C1-Slp2-a (green), and cultured for 48 hours. The cells were re-plated on 35-mm glass-bottomed dishes, cultured for 24 hours, and then stained with Texas Red-conjugated phalloidin (red). Nuclei were stained with DAPI (blue). Scale bars, 10 μm. (Top left) Localization of Slp2-a near the plasma membrane (within 1.5 μm from the plasma membrane) was quantified by using ImageJ software. The fluorescence signals near the plasma membrane were quantified as the ratio of fluorescence intensity near the plasma membrane to total fluorescence intensity, and the ratios were plotted against cell size. (Top right) Total fluorescence intensity was plotted against cell size. Red triangles, control GFP-expressing MDCK II cells (the plasma membrane-localization of GFP was approximately 30%); open circles and closed circles, Slp2-a-overexpressing MDCK II cells, in which the proportion of plasma membrane-localized Slp2-a was more than 30% and less than 30%, respectively. (B) Co-expression of Rap1GAP2 with Slp2-a. (Right) MDCK II cells were transfected with pEGFP-C1-Slp2-a (green) and pmStr-N1-Rap1GAP2 (red), and their localizations and cell sizes were determined as described above. (Left) Slp2-a and Rap1GAP2 exhibited similar localization patterns.
in MDCK II cells. Rap1GAP2, on the other hand, was mainly localized at the plasma membrane and did not affect cell size, when Rap1GAP2 alone was expressed in control MDCK II cells (red triangles).

**Figure 8:** Increased Slp2-a expression and Ezrin activity in the kidneys of *pcy* mice. (A) Kidneys of WT and *pcy* mice. Scale bars, 1 cm. (B) Immunohistochemical staining with anti-Slp2-a antibody (red) and anti-claudin-2 antibody (green) in the kidneys of WT and *pcy* mice. Scale bars, 50 µm. (C) Expression of the Slp family members in the kidneys of WT and *pcy* mice. The lysates of kidneys of WT and *pcy* mice were analyzed by immunoblotting with the antibodies indicated. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (D) Expression of *Slp1*, *Slp2-a*, *Rap1GAP1*, and *Rap1GAP2* in the kidneys of WT and *pcy* mice as revealed by RT-PCR analysis. The size of the molecular weight markers (kbp) is shown on the left. (E) Activity of the EGFR–MAPK, cAMP–PKA, and ezrin–PI3K/AKT pathways in the kidneys of WT and *pcy* mice. The asterisk indicates nonspecific signals. (F, G) Immunohistochemical staining with anti-P-ERM antibody (green) and anti-ezrin antibody (red) (F), and with anti-Slp2-a antibody (green) and anti-P-ERM (red) antibody (G) in the kidneys of WT and *pcy* mice. P-ezrin (P-ERM) was localized at the brush border (apical membrane) of the epithelial cells of the renal proximal tubules in both the WT (top) and *pcy* mice (middle). P-ezrin (P-ERM) was also localized at the basolateral membrane of the renal cyst epithelium in the *pcy* mice (bottom). The asterisks and arrowheads in the kidneys of the *pcy* mice indicate the cyst and ezrin localized at the basolateral membrane, respectively. Scale bars, 50 µm.
Figure 4, Top
Yasuda & Fukuda, Figure 5, Top ↑
Yasuda & Fukuda, Figure 6, Top ↑
A

Plasma membrane total signals (%)

Cell size (x10^3 μm^2)

GFP

GFP-Slp2-a (> 30%)

GFP-Slp2-a (< 30%)

Fluorescence intensity (arbitrary units)

Cell size (x10^3 μm^2)

GFP

Cell size

450 μm^2

400 μm^2

2000 μm^2

4500 μm^2

actin

B

GFP-Slp2-a

GFP-Slp2-a + Rap1GAP2-mStr

Cell size

400 μm^2

2000 μm^2

4500 μm^2

Rap1GAP2-mStr

Rap1GAP2-mStr only

Rap1GAP2-mStr (Slp2-a; > 30%) 

Rap1GAP2-mStr (Slp2-a; < 30%)

Merge

Yasuda & Fukuda, Figure 7, Top ↑