Na,K-ATPase β-subunit *cis* homo-oligomerization is necessary for epithelial lumen formation in mammalian cells

Sonali P. Barwe[1], Anna Skay[2], Ryan McSpadden[1], Thu P. Huynh[1], Sigrid A. Langhans[1], Landon J. Inge[2] and Ayyappan K. Rajasekaran[1],*

[1]Nemours Center for Childhood Cancer Research, Alfred I. duPont Hospital for Children, Wilmington, DE 19803, USA
[2]Pathology and Laboratory Medicine, University of California, Los Angeles, CA 90095, USA
*Author for correspondence (araj@medsci.udel.edu)

Summary

Na,K-ATPase is a hetero-oligomer of an α- and a β-subunit. The α-subunit (Na,K-α) possesses the catalytic function, whereas the β-subunit (Na,K-β) has cell-cell adhesion function and is localized to the apical junctional complex in polarized epithelial cells. Earlier, we identified two distinct conserved motifs on the Na,K-β_1 transmembrane domain that mediate protein-protein interactions: a glycine zipper motif involved in the *cis* homo-oligomerization of Na,K-β_1 and a heptad repeat motif that is involved in the hetero-oligomeric interaction with Na,K-α_1. We now provide evidence that knockdown of Na,K-β_1 prevents lumen formation and induces activation of extracellular regulated kinases 1 and 2 (ERK1/2) mediated by phosphatidylinositol 3-kinase in MDCK cells grown in three-dimensional collagen cultures. These cells sustained cell proliferation in an ERK1/2-dependent manner and did not show contact inhibition at high cell densities, as revealed by parental MDCK cells. This phenotype could be rescued by wild-type Na,K-β_1 or heptad repeat motif mutant of Na,K-β_1, but not by the glycine zipper motif mutant that abrogates Na,K-β_1 *cis* homo-oligomerization. These studies suggest that Na,K-β_1 *cis* homo-oligomerization rather than hetero-oligomerization with Na,K-α_1 is involved in epithelial lumen formation. The relevance of these findings to pre-neoplastic lumen filling in epithelial cancer is discussed.

Key words: Na,K-ATPase, Lumen, Epithelial

Introduction

Na,K-ATPase is a well-characterized oligomeric enzyme comprising two essential subunits, the catalytically active alpha-subunit (Na,K-α_1) and the regulatory beta-subunit (Na,K-β_1). It catalyzes an ATP-dependent transport of three sodium ions out and two potassium ions into the cell, and plays a crucial role in kidney function. Besides its role in maintaining ion homeostasis, Na,K-ATPase performs multiple functions, either dependent or independent of the pump activity (Rajasekaran et al., 2005).

Studies from our laboratory have demonstrated that Na,K-ATPase plays a fundamental role in the formation and maintenance of epithelial polarity in mammalian cells in culture (Rajasekaran et al., 2001b; Rajasekaran et al., 2001a; Rajasekaran et al., 2003; Rajasekaran et al., 2007; Rajasekaran and Rajasekaran, 2009). Repletion of Na,K-β_1 in virally transformed MDCK cells induced tight junctions and polarity (Rajasekaran et al., 2001b). Immunogold electron microscopy studies have shown that Na,K-β_1 is localized to the apical junctional complex in mammalian cells (Rajasekaran et al., 2007). Other studies have replicated these findings in multiple systems. Drosophila homologs of Na,K-α and Na,K-β are exclusively localized to the septate junctions (structures with functional and molecular similarities to vertebrate tight junctions) and are essential for their function (Genova and Fehon, 2003; Paul et al., 2003; Rajasekaran et al., 2008). Na,K-β_1 was required

to maintain normal Na,K-ATPase distribution and localization of tight junction-associated proteins during blastocyst formation in the mouse embryo (Madan et al., 2007). These studies demonstrated that Na,K-β_1 is involved in the assembly of the apical junctional complex and the induction of polarity in epithelial cells.

Na,K-β_1 also functions as a cell adhesion molecule (Rajasekaran et al., 2001a; Kitamura et al., 2005; Shoshani et al., 2005; Vagin et al., 2006; Barwe et al., 2007; Bab-Dinitz et al., 2009). The transmembrane region of Na,K-β_1 consists of two conserved motifs: the heptad repeat motif that is essential for hetero-oligomerization with Na,K-α_1 and the glyzine zipper motif involved in Na,K-β_1 *cis* homo-oligomerization (Barwe et al., 2007). Other studies have shown that N-glycosylation of the extracellular domain of Na,K-β_1 is also involved in its trans-homo-oligomerization and cell-cell adhesion function (Vagin et al., 2006; Tokhtaeva et al., 2011).

Epithelial cells, unlike mesenchymal cells, are polarized into distinct apical and basolateral plasma membrane domains. During organogenesis hollow epithelial structures (called cysts) are formed from mesenchymal cells. These cysts can be regarded as the basic building blocks for the formation of more complex epithelial organs including the kidney. Thus, epithelial lumen formation constitutes a basic architectural characteristic of all organs and is indispensable for their function. To study the molecular mechanisms involved in epithelial lumen formation, a

three-dimensional (3D) culture system mimicking the growth of cells in an extracellular matrix (ECM) environment has been employed (Zegers et al., 2003; Schlüter and Margolis, 2009). Epithelial cells such as Madin-Darby canine kidney (MDCK) cells when cultured in 3D gels of the ECM self-organize into hollow spheres formed by a monolayer of polarized epithelial cells, with an apical surface facing the central lumen, a lateral surface contacting adjacent cells, and a basal surface adhering to the ECM (Zegers et al., 2003). Lumen formation is achieved in three consecutive steps: (1) extracellular matrix and cell-cell recognition, (2) apical-basal polarization and (3) lumen expansion (Datta et al., 2011). In MDCK cells cultured in collagen, apical-basal polarization is achieved by cavitation, i.e. clearing of the non-ECM contacting inner cells by apoptosis. On the other hand, Matrigel grown MDCK cysts undergo apical-basal polarization by hollowing, i.e. generation of luminal space by apical membrane transport to a common point between contacting cells. Altered expression of proteins involved in cell adhesion [e.g. E-cadherin (Desclozeaux et al., 2008; Jia et al., 2011)] or induction of polarity [e.g. Crumbs3 (Roh et al., 2003)] results in lumen formation defects involving multiple lumens or lumen filling. Although there are several evidences indicating a role for Na,K-β_1 in cell adhesion, establishment of the apical junctional complex and polarity in a variety of systems, the role of Na,K-β_1 in epithelial lumen formation has not been investigated.

ERK1 and ERK2 belong to the family of mitogen-activated protein kinases (MAPKs) that are activated by a variety of mitogens, differentiation factors and stress signals. Downregulation of ERK1/2 signaling in transformed MDCK cells restored epithelial morphology and tight junctions (Chen et al., 2000; Schramek et al., 2003). Sustained expression of constitutively active mutant of the ERK activator, MEK1, hindered lumen formation in collagen grown MDCK cysts (Montesano et al., 1999). Knockdown of Na,K-β_1 results in activation of ERK1/2 in porcine kidney epithelial cells (Rajasekaran et al., 2010). Also, ectopic expression of Na,K-β_1 in transformed kidney epithelial cells resulted in suppression of ERK1/2 activation (Inge et al., 2008). Thus, there is an inverse correlation between Na,K-β_1 expression and the level of activated ERK1/2. However, the physiological role of ERK1/2 activation following loss of Na,K-β_1 in epithelial cells is not known.

In this study, using RNA interference, we show that reduced Na,K-β_1 protein levels hindered 3D lumen formation in MDCK cells. Mechanistically, we show that *cis* homo-oligomerization of Na,K-β_1 mediated by the transmembrane domain glycine zipper motif is essential for the lumen formation. Furthermore, we show that activation of ERK1/2 mediated by phosphatidylinositol 3-kinase (PI3-kinase) in Na,K-β_1 knockdown cells plays an important role in suppressing contact inhibition and lumen filling in collagen grown MDCK cells.

Results
Na,K-β_1 is required for lumen formation in MDCK cells
MDCK cells when grown in the presence of collagen form hollow 3D cysts which represent *in vivo* epithelial tissue architecture. We utilized RNA interference to knockdown Na,K-β_1 in MDCK cells to investigate whether Na,K-β_1 is essential for the formation of cysts. MDCK cells stably transduced with retrovirus harboring shRNA against Na,K-β_1

(β-KD) showed 85% reduction in Na,K-β_1 protein levels. Expression of a shRNA resistant mutant of Na,K-β_1 bearing silent mutations within the shRNA recognition site (wild-type rescue construct, β-KD/WT-R) resulted in the restoration of protein levels (Fig. 1A). In collagen gels, the parental MDCK cells formed polarized epithelial structures containing a central lumen with distinct apical and basolateral membrane domains (Fig. 1B,C). By contrast, 74% of β-KD cysts displayed filled lumens containing cells (Fig. 1D). Although the apical junctional actin ring was distinct in control cells, there was a relocalization of this actin staining to basolateral membranes (arrows) in β-KD cells (Fig. 1B,C). β-KD/WT-R cells showed a significant reduction in the number of cysts with filled lumens compared to β-KD cysts (25%, $P<0.005$). Cysts from MDCK and β-KD/WT-R cells showed basolateral localization of the endogenous and overexpressed Na,K-β_1 respectively (Fig. 1B, arrowheads). β-KD cysts had undetectable levels of Na,K-β_1 as revealed by immunofluorescence (Fig. 1B) and by immunoblot analysis (see later). These results indicate that Na,K-β_1 function is essential for epithelial lumen formation in MDCK cells cultured in collagen.

Na,K-β_1 transmembrane domain glycine zipper motif is necessary for lumen formation
In our previous study we showed that Na,K-β_1 transmembrane domain consists of two distinct conserved motifs: a glycine zipper motif involved in the *cis* homo-oligomerization of Na,K-β_1 and a heptad repeat motif that is involved in the hetero-oligomeric interaction with Na,K-α_1. Point mutations in the heptad repeat motif (Y43A/F50A) decreased Na,K-β_1 binding to Na,K-α_1 and reduced Na,K-ATPase pump activity by 40%. Mutation of the glycine zipper motif (G48L) affected Na,K-β_1 *cis* homo-oligomerization and cell-cell adhesion function but did not affect pump activity (Barwe et al., 2007). These mutants were rendered shRNA resistant and transfected into β-KD cells to test whether they rescue the lumen formation phenotype compared to wild-type Na,K-β_1. The expression levels of the Na,K-β_1 mutant and Na,K-α_1 proteins were determined in total protein lysates, and their plasma membrane expression was detected by a cell surface biotinylation assay. The cell surface expression of Na,K-β_1 and Na,K-α_1 in β-KD was reduced by 83% and 61% respectively as compared to the parental MDCK cells (Fig. 2A). The levels of Na,K-β_1 and Na,K-α_1 on the cell surface in β-KD/Y43A/F50A-R cells were reduced by 19% and 32% (Fig. 2A) owing to the compromised interaction between Na,K-α_1 and Na,K-β_1 as described previously (Barwe et al., 2007). Immunofluorescence analysis revealed reduced surface staining of Na,K-α_1 in β-KD and β-KD/Y43A/F50A-R cells (supplementary material Fig. S1). We also observed a 40% reduction in Na,K-ATPase pump activity in β-KD cells compared to MDCK cells (Fig. 2B). The activity was restored in β-KD/WT-R cells. Consistent with our published results (Barwe et al., 2007), β-KD/Y43A/F50A-R cells showed reduced pump activity as compared to the β-KD/G48L-R cells (Fig. 2B).

Phenotypically β-KD cells displayed a fibroblastic morphology in sub-confluent cultures, while the parental cells showed the cobblestone like compacted growth characteristics typical of well-differentiated epithelial cells. This growth characteristic was rescued by β-KD/WT-R and β-KD/Y43A/F50A-R constructs but not by β-KD/G48L-R cells (Fig. 2C). Furthermore, the levels of E-cadherin, a protein that plays an important role in the induction of cell-cell adhesion and epithelial
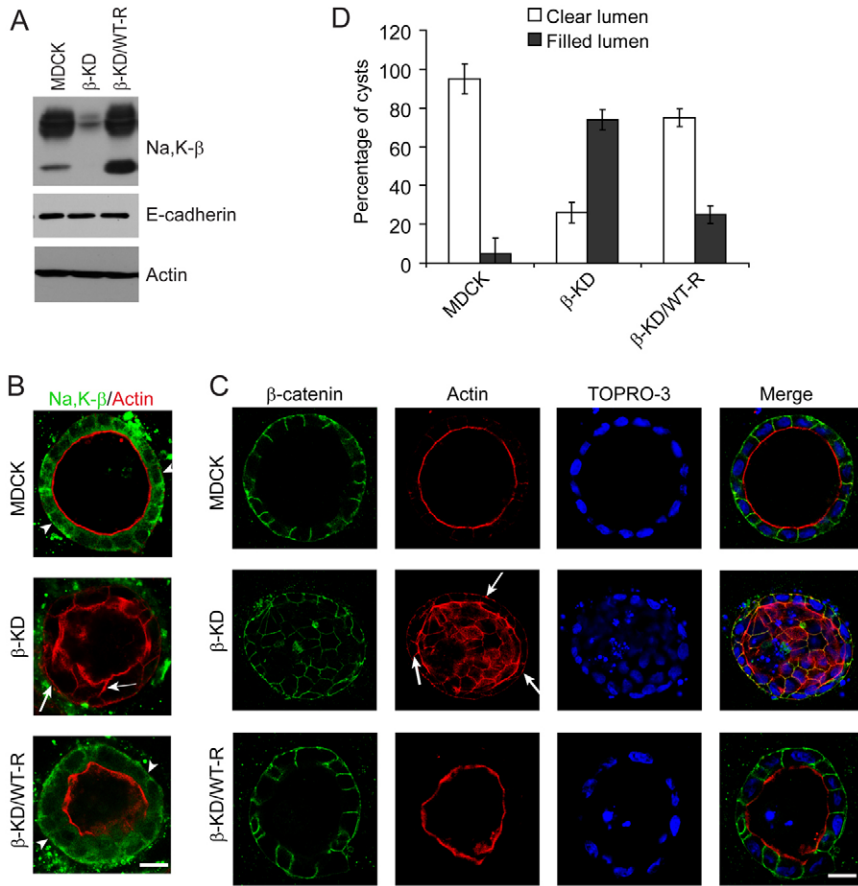
Fig. 1. shRNA-mediated knockdown of Na,K-β_1 in MDCK cells disrupts lumen formation in 3D collagen gels. (**A**) Western blot analysis showing the levels of Na,K-β_1 and E-cadherin from lysates of parental MDCK, β-KD and β-KD/WT-R cells. Actin was used as a loading control. (**B**) Localization of Na,K-β_1 in cysts (arrowheads). Representative images are shown. Note the absence of Na,K-β_1 staining on cell membranes in β-KD cyst. The arrows show the aberrant localization of actin on basolateral cell surface in β-KD cysts. The anti-Na,K-β_1 antibody used for the immunofluorescence analysis gives weak and hazy staining in cysts fixed with paraformaldehyde. (**C**) Parental MDCK, β-KD and β-KD/WT-R cells were cultured in collagen gels for 14 days and stained for β-catenin (basolateral marker; green), actin (apical marker; red), and TOPRO-3 (nuclear marker; blue). Representative images are shown. (**D**) One hundred cysts or cell clusters from each cell-type were analyzed for the presence of clear (or almost clear) and filled lumens. Note that 74% of cell clusters in β-KD cells showed filled lumens. Error bars denote s.d. of the mean from three independent experiments. Scale bars: 20 μm.

polarity (Gumbiner et al., 1988), remained unchanged in β-KD cells (Fig. 1A). These results suggest that the phenotypic change in β-KD cells is likely due to reduced Na,K-β_1-mediated cell adhesion.

In 3D collagen gels, β-KD/Y43A/F50A-R cells were able to form cysts with clear lumens (Fig. 3A). In contrast, β-KD/G48L-R cells showed filled lumen (77%) (Fig. 3A,B). The Na,K-β_1 mutant proteins were expressed at levels comparable to MDCK cells and were localized similarly to the basolateral surface in 3D cysts (Fig. 3C). These results indicated that partial inhibition (40%) of pump activity does not affect lumen formation in β-KD/Y43A/F50A-R cells and that Na,K-β_1 *cis* homo-dimerization mediated by the glycine zipper motif is necessary for this function.

Sustained activation of ERK1/2 prevents lumen formation in Na,K-β_1 knockdown cells

We observed earlier that transient knockdown of Na,K-β_1 induced ERK1/2 activation in porcine kidney epithelial cells (Rajasekaran et al., 2010). Therefore, we tested whether ERK1/2 was activated in β-KD cysts. The levels of phosphorylated ERK1/2 were fourfold higher in β-KD cyst lysates as compared to those from parental MDCK cells (Fig. 4A). Activation of ERK1/2 is specific to the loss of Na,K-β_1 since β-KD/WT-R cells showed near complete reversal of ERK1/2 phosphorylation. The β-KD/Y43A/F50A-R cells showed reduced phospho ERK1/2 levels. By contrast, the β-KD/G48L-R cysts exhibited sustained activation of ERK1/2 indicating that loss of Na,K-β_1 *cis* homo-dimerization mediated by the glycine zipper motif resulted in ERK1/2 activation.

Treatment of β-KD cells with 5 μM PD98059 or U0126 for 48 hours almost completely inhibited ERK1/2 activation as revealed by immunoblot analysis of the phospho ERK1/2 levels (supplementary material Fig. S2A). To test whether activation of ERK1/2 is involved in lumen filling, ERK1/2 was inhibited by PD98059 or U0126 in 7-day-old cysts generated from β-KD cells. This inhibition resulted in the formation of cysts with clear lumens (Fig. 4B–E) in β-KD cells indicating that activation of ERK1/2 following the downregulation of Na,K-β_1 plays an important role in lumen filling in collagen grown β-KD cells. In addition, PD98059 or U0126 treatment also reverted the morphology of β-KD cells from elongated to compact growth phenotype, where as MDCK cells had no effect (supplementary material Fig. S2B). Quantification of cell length using immunofluorescence images of cells stained with anti-β-catenin antibody revealed that β-KD cells were twice longer compared to MDCK or ERK1/2 inhibited β-KD cells (supplementary material Fig. S2C,D). Furthermore, PD98059 inhibited ERK1/2 activation in β-KD/G48L-R cells (supplementary material Fig. S2E) and resulted in cysts with clear lumens (supplementary material Fig. S2F), indicating that activation of ERK1/2 also plays an important role in lumen filling in these cysts.

We further tested whether other mitogen activated protein kinases such as JNK and p38 MAPK were activated in β-KD cells in addition to ERK1/2. There was no change in the phospho JNK and p38 MAPK levels in β-KD cells compared to MDCK cells suggesting that ERK1/2 is specifically activated in β-KD cells (supplementary material Fig. S3A). Furthermore, inhibition of p38 MAPK by treatment with 10 μM SB203580 did not alter
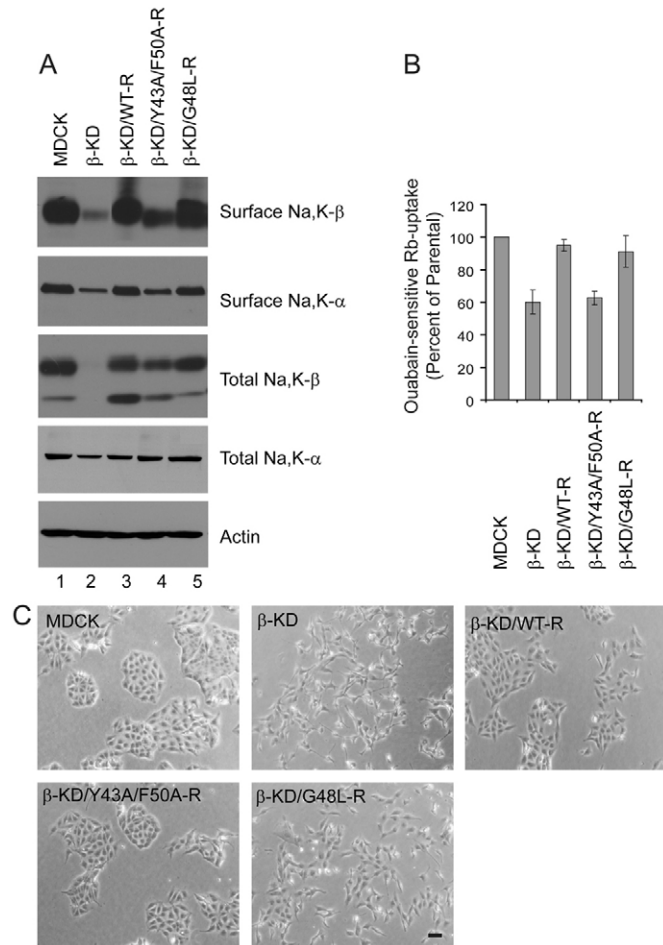
Fig. 2. Na,K-β₁ glycine zipper motif is necessary for compact growth characteristics. (**A**) Western blots showing biotinylated Na,K-α_1 and Na,K-β_1 on the cell surface, and Na,K-α_1, Na,K-β_1 and actin from total lysates of indicated cell types. Representative blots from four independent experiments are shown. (**B**) Rubidium uptake assay to determine the ouabain-sensitive Na,K-ATPase activity in MDCK, β-KD and β-KD cells overexpressing wild-type or mutant Na,K-β_1. Data are presented as mean ± s.e.m. from three independent experiments done in triplicates. (**C**) Morphology of parental MDCK, β-KD and β-KD cells overexpressing wild-type or mutant Na,K-β_1. Representative images are shown. Note the fibroblastic appearance and less compacted growth of β-KD and β-KD/G48L-R cells. Scale bar: 25 μm.

lumen morphology in β-KD cells (supplementary material Fig. S3B).

ERK1/2-mediated suppression of contact inhibition in Na,K-β₁ knockdown cells

Either increased cell proliferation and/or reduced apoptosis could contribute to lumen filling in MDCK cysts (Debnath et al., 2002). To test the role of Na,K-β_1 in apoptosis, we subjected MDCK and β-KD cells to serum starvation and performed a DNA fragmentation assay (Zhu et al., 1996). When deprived of serum, MDCK and β-KD cells showed DNA laddering due to apoptosis (supplementary material Fig. S4), suggesting that knockdown of Na,K-β_1 did not protect the cells from apoptosis under serum starvation and that reduced apoptosis is likely to play a minimal role in lumen filling in β-KD cells.

ERK1/2 activation is known to suppress contact inhibition of cell proliferation in MDCK cells and its phosphorylation levels decline as cells attain higher densities (Li et al., 2004). We hypothesized that increased ERK1/2 levels in β-KD cells are associated with enhanced cell proliferation and suppression of contact inhibition. To test this hypothesis we determined the phospho-ERK1/2 levels in β-KD and rescue cells grown at low and high densities. At low densities, all cell lines had higher levels of phospho-ERK1/2 (Fig. 5A). At high densities, ERK1/2 phosphorylation was downregulated in MDCK, β-KD/WT-R and β-KD/Y43A/F50A-R cells, whereas β-KD and β-KD/G48L cells retained high phospho-ERK1/2 levels. ERK1/2 positively regulates cyclin D1 (Lavoie et al., 1996) and cyclin D1 accumulation is a marker for entry of MDCK cells into the cell cycle (Marschitz et al., 2000). Consistent with increased phospho-ERK1/2 levels, cyclin D1 levels were elevated in β-KD and β-KD/G48L-R cells suggesting that these cells continue to proliferate even at higher densities.

A colorimetric bromodeoxyuridine (BrdU) uptake assay was utilized to monitor the proliferation of cells at low and high densities. β-KD and β-KD/G48L-R cells showed 2.5-fold higher BrdU uptake as compared to the parental MDCK cells at low densities (Fig. 5B). The β-KD/WT-R and β-KD/Y43A/F50A-R cells showed similar BrdU incorporation like parental MDCK cells. At higher densities (3 days of growth) MDCK cells showed minimal BrdU uptake due to the contact inhibition of cell growth (Liu et al., 2010). However, β-KD and β-KD/G48L-R cells at higher densities showed 2- to 2.5-fold higher BrdU incorporation compared to β-KD/WT-R and β-KD/Y43A/F50A-R cells suggesting that ERK1/2 is involved in the continued cell proliferation of β-KD and β-KD/G48L-R cells. To test the role of ERK1/2 in suppression of contact inhibition, we treated MDCK and β-KD cells at low and high densities with PD98059. Treatment with PD98059 at low cell densities reduced BrdU incorporation in both MDCK and β-KD cells (Fig. 5C). At higher densities, MDCK cells showed minimal BrdU uptake which did not change in the presence of PD98059. By contrast, β-KD cells maintained BrdU uptake at high densities, and PD98059 treatment reduced its uptake by about 62.5%. These results strongly suggest that activated ERK1/2 is involved in the sustained cell proliferation and suppression of contact inhibition in β-KD cells.

PI3 kinase is involved in the activation of ERK1/2 in β-KD and β-KD/G48L-R cells

In order to identify the signaling mechanism by which ERK1/2 is activated in β-KD cells, cells were treated with inhibitors of key signaling proteins such as PI3-kinase (10 μM LY294002), epithelial growth factor receptor (EGFR) (10 μM AG1478, 1 μM EKB-569), MEK1/2 (10 μM PD98059), Ras (10 μM farnesylthiosalicylic acid, FTS) and protein kinase C (PKC) (10 μM bisindolylmaleimide, BIM). Inhibition of MEK1/2, the upstream activator of ERK1/2, suppressed ERK1/2 phosphorylation as expected. EGFR, Ras and PKC inhibitors did not attenuate ERK1/2 phosphorylation, while LY294002 suppressed ERK1/2 phosphorylation by 63% ($P=0.003$, Fig. 6A). To further support the involvement of PI3-kinase in ERK1/2 activation in β-KD cells, we tested the phosphorylation status of downstream effectors of PI3-kinase such as Akt, p70 S6 kinase and glycogen synthase kinase-3β (GSK-3β). The levels of phosphorylated Akt, p70 S6 kinase and GSK-3β were higher in
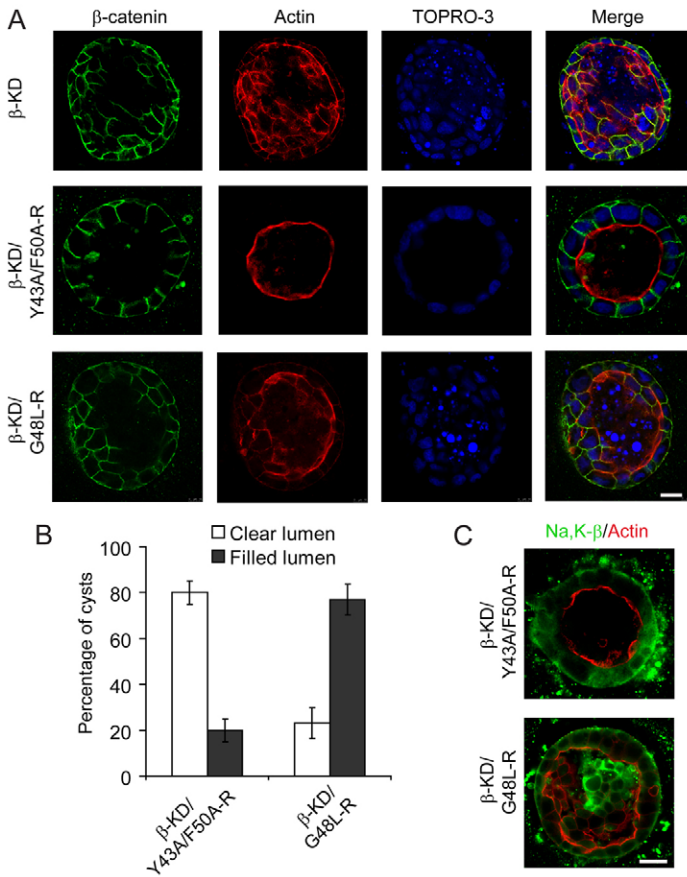
Fig. 3. **Lumen formation in β-KD cells rescued by expression of Na,K-β₁ transmembrane domain mutants.** (**A**) Cysts from β-KD, β-KD/Y43A/F50A-R or β-KD/G48L-R cells cultured in 3D collagen gels were stained with anti-β-catenin antibody (green), phalloidin-Alexa Fluor 594 (for actin, red), and TOPRO-3 (for nuclei, blue). Note that β-KD and β-KD/G48L-R cysts fail to form clear lumens. (**B**) One hundred cysts or cell clusters from each cell-type were analyzed for the presence of clear (or almost clear) and filled lumens. Error bars denote s.d. of the mean from three independent experiments. (**C**) Localization of mutant Na,K-β₁ in β-KD/Y43A/F50A-R and β-KD/G48L-R cysts. Representative images are shown. Scale bars: 20 μm.

β-KD cells as compared to MDCK cells and were reduced in β-KD/WT-R cells, suggesting that PI3-kinase is specifically activated following knockdown of Na,K-β₁ (supplementary material Fig. S5).

LY294002 also suppressed ERK1/2 phosphorylation in β-KD/G48L-R cells by 62% (supplementary material Fig. S2E). These results suggest the involvement of PI3-kinase in the activation of ERK1/2 in β-KD and β-KD/G48L-R cells. To test this possibility, 7-day-old β-KD and β-KD/G48L-R cysts were treated with 10 μM LY294002 for 7 days. The treated cysts showed partial restoration of lumen formation (66–72%) (Fig. 6B,C). Taken together, these data indicate that PI3-kinase is responsible at least in part for the activation of ERK1/2 leading to lumen filling in β-KD and β-KD/G48L-R cysts.

Discussion

In the past decade several studies from our laboratory and others revealed novel functions of Na,K-β₁. Specifically, these studies focused on its role in epithelial polarity and cell adhesion. In this study, we provide the first evidence that Na,K-β₁ is essential for lumen formation in MDCK cells grown in a 3D collagen matrix. Mechanistically, we show that reduced expression or loss of *cis* homo-oligomerization of Na,K-β₁ is associated with PI3-kinase-mediated ERK1/2 activation leading to suppression of contact inhibition and lumen filling. These studies suggest that Na,K-β₁ homo-oligomerization rather than hetero-oligomerization with the Na,K-α₁ is involved in epithelial lumen formation.

Knockdown of Na,K-β₁ resulted in reduced Na,K-ATPase activity, altered compact growth characteristics in 2D culture and

affected lumen formation in 3D culture. β-KD cells also showed enhanced ERK1/2 activation. The wild-type Na,K-β₁ construct rescued Na,K-ATPase pump activity, the less compact growth phenotype, lumen formation, and reduced ERK1/2 activation. These results showed that the observed multiple effects are specific to the expression of wild-type Na,K-β₁.

Ion transport function of Na,K-ATPase has been shown to regulate single lumen formation. Inhibition of Na,K-ATPase pump function in zebrafish brain ventricles (Zhang et al., 2010) and gut (Bagnat et al., 2007), MDCK (Bagnat et al., 2007), and Caco-2 cells (Jaffe et al., 2008) cultured in Matrigel affected lumen formation. Pump inhibition led to compromised lumenal fluid accumulation, resulting in reduced lumenal expansion and smaller lumen size (Bagnat et al., 2007). The β-KD cells did not display smaller lumen, probably because significant level of pump activity (60%) was present. However, the lumen formation was affected. Our results are consistent with the idea that the Na,K-β₁ homo-oligomerization-mediated cell adhesion is also required for lumen formation. Because, (1) Rescue of the β-KD cells with heptad repeat mutant construct (β-Y43A/F50A) did not rescue the pump activity. Yet β-KD/Y43A/F50A-R cells developed lumen similar to MDCK cells. Therefore, a decrease in enzyme activity observed in these cells did not affect lumen formation. (2) Rescue of β-KD cells with the glycine zipper mutant construct (β-G48L) restored the enzyme activity in β-KD/G48L-R cells. Yet these cells did not form lumen suggesting that reduced enzyme activity in β-KD cells has a minimal effect on lumen formation. Thus, rescue experiments with two different mutants showed that lumen formation was dependent on the cell
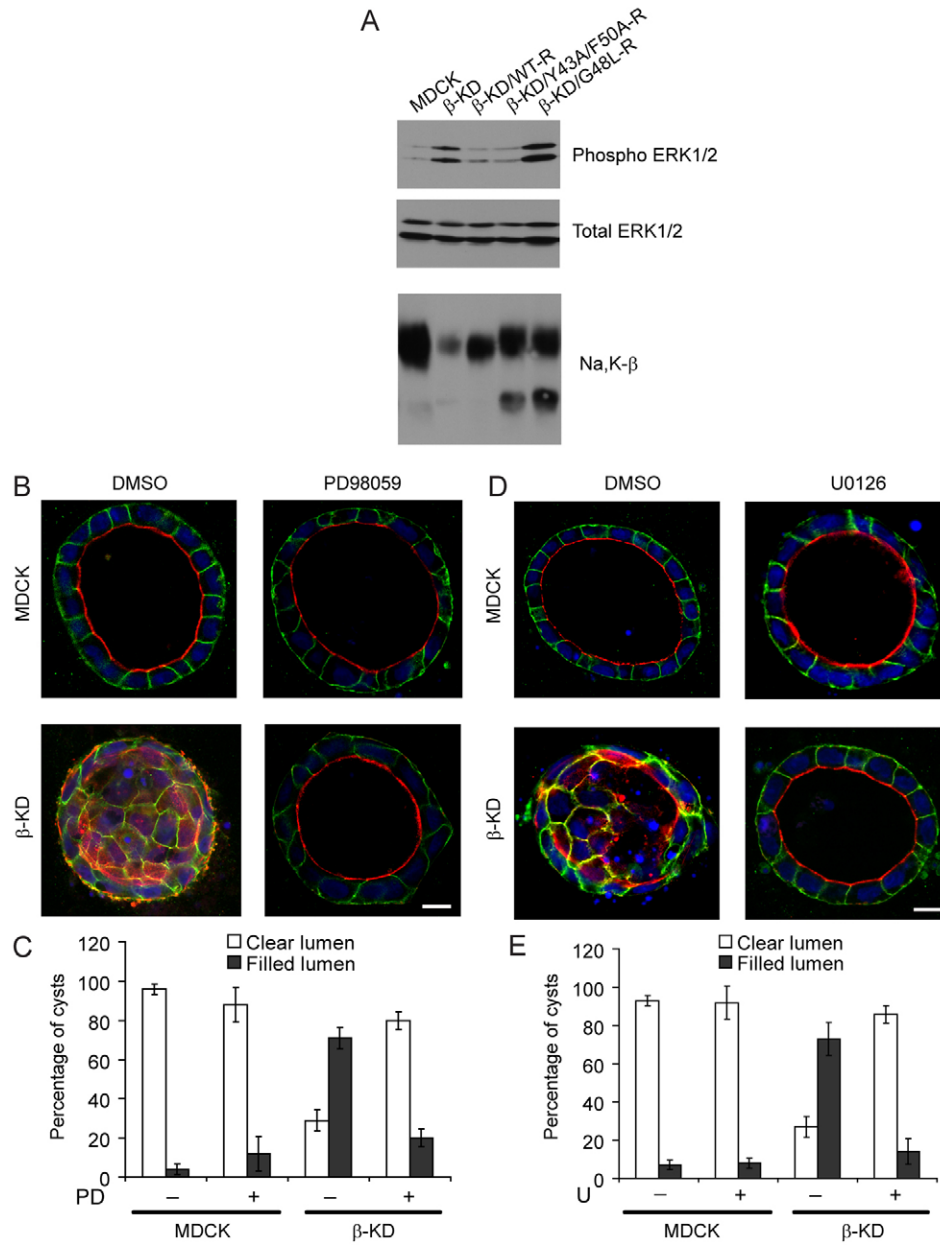
Fig. 4. β-KD cells show sustained ERK1/2 activation, and inhibition of ERK1/2 suppresses lumen filling in β-KD cysts. (**A**) Western blots showing levels of phosphorylated and total ERK1/2 and of Na,K-β₁ in cell lysates from 3D cysts cultured in collagen for 14 days. Representative blots from three independent experiments are shown. (**B–E**) Cells were cultured in 3D collagen gels for 7 days. At this time, cysts were treated with 5 µM PD98059 (B,C) or 5 µM U0126 (D,E) to inhibit ERK1/2 activation. (B,D) Merged images obtained from staining cysts with anti-β-catenin antibody (green), phalloidin-Alexa Fluor 594 (for actin, red) and TOPRO-3 (for nuclei, blue) are shown. Note that PD98059 or U0126 treatment did not affect MDCK cysts; however, it aided β-KD cells in the formation of cysts with clear lumens. (C,E) One hundred cysts or cell clusters from each cell-type were analyzed for the presence of clear (or almost clear) and filled lumens. Error bars denote s.d. of the mean from three independent experiments. Scale bars: 20 µm.

adhesion mediated by the glycine zipper motif rather than pump activity.

Although β-levels were reduced by 90% in β-KD cells, the Na,K-α₁ levels were reduced by only 40% and significant levels of Na,K-α₁ were present at the plasma membrane. We have reported similar findings in cancer cells where significant levels of Na,K-α₁ and pump activity were retained even if the Na,K-β₁ levels were reduced more than 80% (Rajasekaran et al., 1999; Espineda et al., 2004; Rajasekaran et al., 2010). In addition, the levels of Na,K-α₁ remained unchanged when Snail1, a transcription suppressor of Na,K-β₁ was expressed in MDCK or MCF7 cells, suggesting that either small amount of Na,K-β₁ is sufficient or other isoforms of Na,K-β₁ are involved in the transport of Na,K-α₁ to the membrane. RT-PCR analysis of Na,K-β₂ and Na,K-β₃, revealed the presence of Na,K-β₂ but not Na,K-β₃ transcripts in MDCK cells (supplementary material Fig. S6A). Western blot analysis

showed a marginal increase in Na,K-β₂ protein levels in β-KD cells compared to MDCK cells (supplementary material Fig. S6B). However, it is unlikely that this Na,K-β₂ contributes to the membrane localization of Na,K-α₁. Moreover, a recent study revealed that Na,K-α₁ preferentially associates with Na,K-β₁ and not Na,K-β₂ (Tokhtaeva et al., 2012).

The Na,K-β₁ cell-cell adhesion function is inversely proportional to the activation of ERK1/2. Increased cell-cell aggregation induced by Na,K-β₁ is associated with reduced ERK1/2 activation suggesting that the cell-cell contact mediated by Na,K-β₁ reduces ERK1/2 activation (Inge et al., 2008). In this study, we showed that stable knockdown of Na,K-β₁ resulted in the sustained activation of ERK1/2. This result is consistent with the ERK1/2 activation in kidney proximal tubule epithelial cells with transient knockdown of Na,K-β₁ (Rajasekaran et al., 2010). Moreover, mutation of the glycine zipper motif which inhibits *cis*
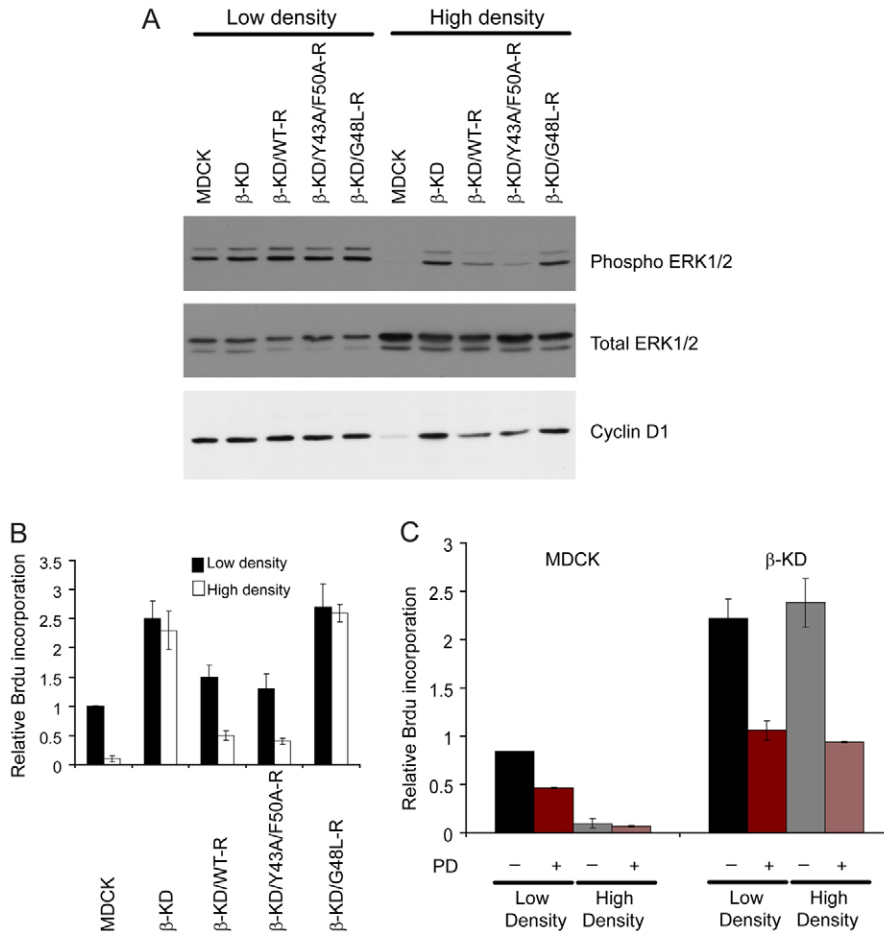
Fig. 5. ERK1/2 inhibition suppresses contact inhibition in β-KD cysts. (**A**) Immunoblots showing levels of phosphorylated and total ERK1/2 and of cyclin D1 from cells cultured at low (<10% confluent) or high (contact-inhibited) densities. Representative blots from three independent experiments are shown. (**B**) Brdu incorporation assay in indicated cells at day 1 (low density) or day 3 (high density) after plating. (**C**) Brdu incorporation assay in MDCK and β-KD cells. Cells were plated at low density and treated the following day with DMSO or 10 µM PD98059 for 12 hours. Another set of cells were plated at a high density and allowed to grow for 3 days before treatment with DMSO or 10 µM PD98059 for 12 hours. Error bars denote s.d. of the mean from three independent experiments.
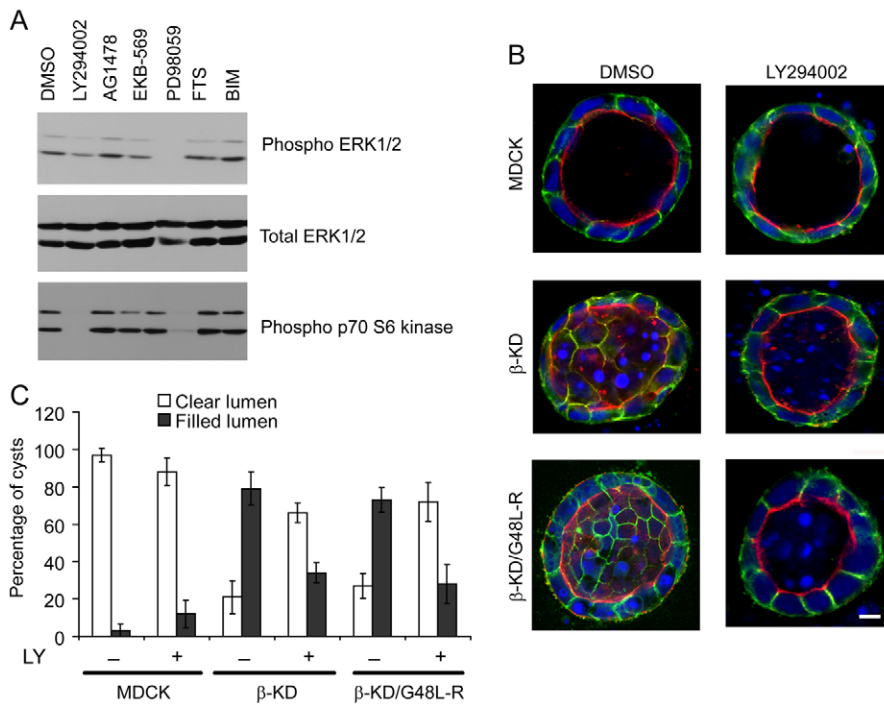

Fig. 6. PI3 kinase is involved in the activation of ERK1/2 in β-KD cells. (**A**) Immunoblots showing levels of phosphorylated and total ERK1/2 and of phosphorylated p70 S6 kinase from β-KD cells treated with inhibitors of different signaling pathways. Phosphorylation of p70 S6 kinase (a downstream effector of PI3 kinase) was inhibited at this concentration of LY294002, confirming that PI3 kinase is inhibited at this concentration. Representative immunoblots from three independent experiments are shown. (**B**) Cells cultured in 3D collagen gels for 7 days were treated with 10 µM LY294002 for 7 additional days. The merged image obtained from staining cysts with anti-β-catenin antibody (green), phalloidin-Alexa Fluor 594 (for actin, red) and TOPRO-3 (for nuclei, blue) is shown. Note that LY294002-treated β-KD and β-KD/G48L-R cysts show nuclear fragments but not intact cells in their lumens. Scale bar: 20 µm. (**C**) One hundred cysts or cell clusters from each cell-type were analyzed for the presence of clear (or almost clear) and filled lumens. Error bars denote s.d. of the mean from three independent experiments.

Journal of Cell Science

homo-oligomerization of Na,K-β_1 also resulted in increased ERK1/2 activation. Taken together, these studies strongly suggest that Na,K-β_1-mediated *cis* homo-oligomerization suppresses ERK1/2 activation. Our results further suggest that PI3-kinase is upstream of ERK1/2 activation in β-KD cells. However, the precise mechanism by which PI3-kinase activates ERK1/2 due to loss of Na,K-β_1 expression is not known.

Lumen formation is achieved by two separate mechanisms depending on the extracellular matrix (ECM) used for 3D culture. Cells grown in collagen (weaker polarization cues) require apoptosis for lumen formation. However, in Matrigel (strong polarization cues), lumen is formed by exocytosis and membrane separation, without the need for apoptosis (Martín-Belmonte et al., 2008). β-KD cells were able to form cysts with clear lumens when cultured in Matrigel (supplementary material Fig. S7) but lumen formation was affected in collagen matrix, suggesting that Na,K-β_1 plays a role in regulating apoptosis and/or cell proliferation. DNA laddering experiment showed that apoptosis was induced by serum starvation in both MDCK and β-KD cells indicating that reduced Na,K-β_1 expression does not protect cells from apoptosis. ERK1/2 phosphorylation has been shown to regulate contact inhibition of proliferation in MDCK cells grown in 2D culture (Li et al., 2004). Our results are consistent these findings in 2D culture. We found that at high cell densities the levels of active ERK1/2 and BrdU incorporation decreased in MDCK cells whereas β-KD cells showed sustained ERK1/2 activation and BrdU uptake. Consistent with this finding in 3D culture ERK inhibitor restored lumen formation in β-KD cells. Therefore, the lumen filling in β-KD cysts appears to be due ERK1/2 activation, which in turn results in the suppression of contact inhibition and increased cell proliferation.

Ouabain has been shown to induce ERK1/2 activation and inhibit apoptosis in HUVEC cells (Trevisi et al., 2004). Although pharmacological inhibition of Na,K-ATPase by ouabain induced lumen filling in collagen grown MDCK cells it did not induce ERK1/2 activation in MDCK cysts (supplementary material Fig. S8). Furthermore, treatment with PD98059 did not restore lumen formation in these cells (supplementary material Fig. S8) indicating that ERK1/2 is minimally involved in lumen filling in ouabain-treated MDCK cells. These results suggest that mechanisms affecting lumen formation in β-KD cells and ouabain-treated MDCK cells are distinct. Future experiments are necessary to clarify these mechanisms.

Early stages of many epithelial cancers display lumenal filling and loss of cyst polarization (Debnath and Brugge, 2005). Na,K-β_1 levels are downregulated in a variety of cancers including clear cell renal cell carcinoma (Rajasekaran et al., 1999). ERK1/2 activation is known to cause lumen filling and preneoplastic growth in carcinoma (Reginato et al., 2005). It is tempting to speculate that downregulation of Na,K-β_1 is an early event in oncogenesis which might result in the activation of ERK1/2 contributing to suppression of contact inhibition and preneoplastic growth. Therefore, understanding the molecular mechanisms leading to loss of Na,K-β_1 and its role in the formation and maintenance of epithelial lumen should provide novel insights in cancer progression.

Materials and Methods
Cell lines, plasmid constructs and DNA transfection
MDCK cells obtained from American Type Culture Collection (Rockville, MD) were maintained in DMEM with 10% fetal bovine serum, 2 mM/L L-glutamine, 25 U/mL penicillin, and 25 µg/mL streptomycin. For stable knockdown of Na,K-β_1, the p*Silencer* 5.1-U6 Retro vector (Ambion, TX) containing appropriate siRNA targeting sequence as described (Rajasekaran et al., 2010) was transfected into MDCK cells. The full-length dog Na,K-β_1 cDNA harboring silent mutations within the shRNA recognition site to make it shRNA-resistant was first constructed. The G48L (for glycine zipper), Y43A/F50A (heptad repeat motif) mutants (described in Barwe et al., 2007) were then introduced within the shRNA-resistant Na,K-β_1 construct. Point mutations were generated using QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following manufacturer's protocol. All mutant constructs were confirmed by sequencing. Transfection was done by electroporation using the Amaxa Nucleofector (Lonza, Walkersville, MD), following the manufacturer's instructions. Cells were maintained in a selection medium for several days post transfection. Pooled stable clones of cells expressing the transfected constructs were utilized for the experiments.

Antibodies
Mouse monoclonal antibodies (mAbs) raised against Na,K-β_1 (M7-PB-E9) and Na,K-α_1 (M17-P5-F11) have been characterized and described previously (Abbott and Ball, 1993; Sun and Ball, 1994), and were kindly provided by Dr William Ball Jr., University of Cincinnati, Cincinnati, OH. Anti-Na,K-β_1 antibody (for staining Na,K-β_1 in cysts from paraformaldehyde-fixed collagen gels) and anti-Na,K-β_2 antibody (for immunoblotting) were kindly provided by Dr Pablo Martin-Vasallo, Universidad de La Laguna, Tenerife, Spain. Antibodies against phospho-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204), p44/p42 MAPK (ERK1/2), cyclin D1, phospho-p70 S6 kinase (Thr389), phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, and phospho-p38 MAPK (Thr180/Tyr182), phospho-Akt (Ser473), and phospho-GSK-3β (Ser9) and horseradish peroxidase conjugated secondary antibodies were obtained from Cell Signaling Technology (Lexington, KY). E-cadherin antibody was obtained from BD Transduction Laboratories (San Jose, CA). Anti-β-actin antibody was purchased from Sigma-Aldrich (St Louis, MO). Alexa-Fluor-488-conjugated anti-mouse secondary antibody, Alexa-Fluor-595-conjugated phalloidin and TOPRO-3 was purchased from Molecular Probes (Eugene, OR).

Immunoblotting
Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM sodium glycerol phosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 5 µg/ml each of antipain, leupeptin and pepstatin. The lysates were sonicated, and clarified by centrifugation at 16,000 *g* for 10 minutes at 4℃. Total protein was estimated from the supernatants using the Bio-Rad DC reagent (Bio-Rad, Hercules, CA) following manufacturer's instructions. Lysates corresponding to equal amounts of total protein were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted using a primary antibody, and HRP-conjugated secondary antibody diluted in Tris-buffered saline containing either 5% (w/v) non-fat dried milk or 5% (w/v) BSA, and 0.1% (v/v) Tween-20. The proteins were detected by using the enhanced chemiluminescent lightning system according to the manufacturer's recommendations (PerkinElmer Life Sciences, Boston, MA).

For density experiments, low density cells (<10% confluence) were seeded on plastic dishes and lysed the following day. Cells were plated at high densities on Transwell filters (0.4 µm pore size) and allowed to grow and polarize for three days as described previously (Li et al., 2004).

Cell surface biotinylation
Cell surface biotinylation was performed as described previously (Barwe et al., 2007), using 0.5 µg/ml of membrane-impermeable EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL). Cells were lysed in 20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% (w/v) BSA, 1 mM PMSF, 5 µg/ml each of antipain, leupeptin and pepstatin. Lysate was precipitated for 16 hours at 4℃ with 30 µl of Ultralink Streptavidin beads (Pierce). The precipitates were washed, loaded onto a gel and immunoblotted as described above.

Rubidium transport assay
The ouabain-sensitive ion transport was estimated by determining the uptake of $^{86}Rb^+$ as described previously (Barwe et al., 2007). The cells were incubated with 50 µM ouabain (Sigma-Aldrich) for 30 minutes at 37℃ before the experiment. Samples were normalized to protein content and the ouabain-sensitive Rb flux was calculated.

3D cysts; culture, lysis and inhibitor treatments
MDCK cysts were grown in 3D collagen gels as described (Elia and Lippincott-Schwartz, 2009). Briefly, MDCK cells were trypsinized, triturated into a single cell suspension, and mixed into an ice-cold solution containing rat tail collagen I, DMEM, 20 mM HEPES pH 7.4 such that the final cell density is (2×10^4 cells/ml). The resulting suspension was added to Transwell membrane filters and allowed to form a solidified gel at 37℃ prior to addition of culture media. The media was replaced every two days for 14 days. At this point, the cysts were fixed with 4%

paraformaldehyde, RNase-treated and stained using anti-β-catenin antibody, phalloidin and TOPRO-3.

Lysates from cysts were generated following a protocol described before (O'Brien et al., 2006). Briefly, cells were plated at a higher density (2×10^5 cells/ml) than used for cyst formation routinely, and placed on top of a thin layer of collagen gel. The cysts were solubilized by boiling the gels for 15 minutes in a buffer containing 0.5% SDS, 100 mM NaCl, 50 mM triethanolamine, pH 8.1, 5 mM EDTA, pH 8.0, 0.2% NaN$_3$. At this point, the lysates were allowed to cool and supplemented with 1 mM PMSF, and 5 μg/ml each of antipain, pepstatin and leupeptin.

Since inhibition of ERK1/2 in growing MDCK cysts greatly reduces their growth (Turner et al., 2007), treatment with MEK inhibitor PD98059 (5 μM) or U0126 (5 μM) was started when cysts were 7 days old and continued every other day until cysts were fixed at 14 days as described previously (Tushir et al., 2010). Similarly, PI3-kinase or p38-MAPK inhibition was achieved by treatment of 7-day-old cysts with 10 μM LY294002 or 10 μM SB203580 respectively for 7 days. For supplementary material Fig. S8, 7-day-old cysts were treated with either DMSO, 0.1 μM ouabain or 0.1 μM ouabain in the presence of 5 μM PD98059 for 7 additional days, at which point the cysts were fixed and immunostained for confocal microscopy. Ouabain concentration of 0.1 μM was consistent with previous reports in MDCK cells (Bagnat et al., 2007).

MDCK cells were cultured in reduced growth factor Matrigel as previously described (O'Brien et al., 2006).

Microscopy and quantitation of 3D cysts

Cysts were imaged by Leica SP5 laser scanning confocal microscope. The presence of cells within the lumen was observed. The number of cysts with clear or filled lumens were counted across at least ten different 40× fields and expressed as a percentage of total number of cysts. At least 100 cysts were examined per experimental group.

For supplementary material Fig. S1, a stack of seven serial sections 0.2 microns apart was combined into a 3D projection. All images were captured using the same laser intensity and gain/offset settings.

Bromodeoxyuridine assay

BrdU cell proliferation assay kit (EMD Millipore, Billerica, MA) was used following manufacturer's instructions. For measurement of cell proliferation at low density cells were plated at <10% confluence on 96-well plates and assayed for Brdu incorporation on the following day. For cell proliferation measurement at high density, cells plated at confluent densities were allowed to grow for three more days and then assayed for BrdU incorporation.

Apoptosis assay

Apoptosis assay was performed as described previously for MDCK cells (Zhu et al., 1996). Briefly, MDCK cells were plated either in DMEM containing 0.1% or 10% serum and cultured for 48 hours. At this time, the floating cells and adherent cells were collected. Genomic DNA isolated from these cells was treated with RNase and resolved on an agarose gel. The appearance of a DNA ladder suggests the occurrence of apoptosis.

RT-PCR

Primer sets used were Na,K-β$_1$ forward, 5′-GGTGGCAGTTGGTTTAAGATCC-3′ Na,K-β$_1$ reverse, 5′-GGAATCTGTGTTAATCCTGG-3′ Na,K-β$_2$ forward, 5′-ACACCGGGCTTGATGATTCG-3′, Na,K-β$_2$ reverse, 5′-AACAAGTTCTTG-GAGCCTTACAACG-3′, Na,K-β$_3$ forward, 5′-GTTCCAAAATACCGTGACC-AGATTCC-3′, Na,K-β$_3$ reverse, 5′-GGAGCACTTTTTGAACAGAAGGGT-3′. Glucose-6-phosphate dehydrogenase (GAPDH) forward, 5′-GCTGTCCAAC-CACATCTCCTC-3′; reverse, 5′-TGGGGCCGAAGATCCTGTT-3′. A representative gel is shown. Total RNA isolated from cells by Trizol was subjected to Reverse Transcription to generate cDNA, which was used as a template to amplify specific DNA fragments using primers described above.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.108795/-/DC1

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