Knockdown of sodium–calcium exchanger 1 induces epithelial-to-mesenchymal transition in kidney epithelial cells

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Mesenchymal-to-epithelial transition (MET) and epithelial-to-mesenchymal transition (EMT) are important processes in kidney development. Failure to undergo MET during development leads to the initiation of Wilms tumor, whereas EMT contributes to the development of renal cell carcinomas (RCC). The role of calcium regulators in governing these processes is becoming evident. We demonstrated earlier that Na+/Ca2+ exchanger 1 (NCX1), a major calcium exporter in renal epithelial cells, regulates epithelial cell motility. Here, we show for the first time that NCX1 mRNA and protein expression was down-regulated in Wilms tumor and RCC. Knockdown of NCX1 in Madin-Darby canine kidney cells induced fibroblastic morphology, increased intercellular junctional distance, and induced paracellularity, loss of apico-basal polarity in 3D cultures, and anchorage-independent growth, accompanied by expression of mesenchymal markers. We also provide evidence that NCX1 interacts with and anchors E-cadherin to the cell surface independent of NCX1 ion transport activity. Consistent with destabilization of E-cadherin, NCX1 knockdown cells showed an increase in β-catenin nuclear localization, enhanced transcriptional activity, and up-regulation of downstream targets of the β-catenin signaling pathway. Taken together, knockdown of NCX1 in Madin-Darby canine kidney cells alters epithelial morphology and characteristics by destabilization of E-cadherin and induction of β-catenin signaling.

Epithelial cells that line the renal tubules possess apico-basal polarity and a defined cell structure maintained by junctional proteins. These junctions enable adhesion with neighboring cells and the basement membrane. The underlying mesenchymal cells, which secrete extracellular matrix proteins, are fibroblastic with a non-uniform cell structure. Mesenchymal-to-epithelial transition (MET)2 is an important process in the induction of nephron from the metanephric mesenchyme, and epithelial-to-mesenchymal transition (EMT) is a common occurrence during renal tubule formation, where the epithelial cells acquire mesenchymal phenotype transitorily before reverting back to the epithelial phenotype. Multiple rounds of MET and EMT occur during kidney development (1). Differentiation arrest of mesenchymal cells results in the genesis of Wilms tumor, or nephroblastoma, which is the most frequently occurring renal cancer in children (2, 3). Aberrant transformation of tubular epithelial cells of the nephron via EMT is associated with the development of renal cell carcinoma (RCC) (4–6), which constitutes 85–90% of the adult renal tubular malignancies (7).

Kidney plays a vital role in maintaining ionic balance in the body. The principal Ca2+ regulator that mediates Ca2+ reabsorption on the basolateral surface of epithelial cells lining the distal convoluted tubules and connecting tubules in a nephron is the Na+/Ca2+ exchanger (NCX1) (8). NCX1 is a major Ca2+ extrusion mechanism in renal epithelial cells, responsible for two-thirds of Ca2+ extrusion (8); isoforms NCX2 and NCX3 are not expressed in these cells (9–11). NCX1 consists of 10 α-helical transmembrane domains and a large intermediate cytosolic loop with 550 residues. This large intracellular loop contains two Ca2+-binding domains, which, along with the catenin-like domain, mediate the ion exchange activity (12). NCX1 has been shown to function in forward mode in renal epithelial cells (i.e. mediate the extrusion of one Ca2+ and the influx of 3 Na+) in one exchange movement (8).

We showed earlier that functional inhibition of NCX1 led to enhanced cell migration in renal epithelial cells and that NCX1 interacts with adhesion protein, the β-subunit of Na,K-ATPase (13). Another study indicated that NCX1 was up-regulated during stroma-induced cell adhesion in the prostate epithelium (14). Because increased migration and suppression of cell—cell

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2 The abbreviations used are: MET, mesenchymal-to-epithelial transition; EMT, epithelial-to-mesenchymal transition; RCC, renal cell carcinoma(s); NCX1, Na+/Ca2+ exchanger 1; TER, transepithelial electrical resistance; ECIS, electrical cell-substrate impedance sensing; TAMRA, 6-carboxytetramethylrhodamine; LIF, lymphoid enhancer-binding factor-1; TCF, T cell factor 3; TRP, transient receptor potential; PMCA, plasma membrane Ca2+-ATPase; KD, knockdown; MDCK, Madin-Darby canine kidney; PI, protease inhibitor; TEA, triethylammonium.

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adhesion is a prerequisite for cancer progression, we determined NCX1 levels in renal cancers and evaluated the role of NCX1 in EMT. This is a first report showing reduced levels of NCX1 in both RCC and Wilms tumor and that knockdown of NCX1 induces EMT in MDCK cells.

Results

Expression of NCX1 mRNA and protein is down-regulated in renal cancers

We showed earlier that inhibition of NCX1 increases cell migration in kidney epithelial cells (13). Because enhanced migration is one of the characteristics acquired by carcinoma cells, we tested whether NCX1 expression is altered in renal cancers. An analysis of publically available microarray data from a genomic study (GSE11151) (15, 16) revealed that NCX1 mRNA levels were reduced in all three subtypes of RCC and in pediatric Wilms tumor compared with normal kidney tissue (Fig. 1A). Similar down-regulation of NCX1 was also observed in other studies (16–18, 81) analyzed on Oncomine (Compen-

Knockdown of NCX1 changes the epithelial morphology of MDCK cells

To determine whether reduced NCX1 expression is instrumental in the development of renal cancers, MDCK cells with stable NCX1 knockdown (NCX1-KD) were generated by transduction of lentiviral particles containing two different shRNA sequences targeted against NCX1. NCX1 protein was reduced by 77% in the cells with NCX1 knockdown (Fig. 2, A and C). Similar down-regulation of NCX1 protein in EMT. This is a first report showing reduced levels of NCX1 in both RCC and Wilms tumor and that knockdown of NCX1 induces EMT in MDCK cells.

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Cysts were comparatively larger than MDCK cysts (Fig. 3, A and C). The 30% increase in cyst dimension could be due to the higher rate of proliferation (1.4-fold) in NCX1-KD cells compared with MDCK cells (Fig. 3D).

**NCX1 regulates the tightness of intercellular junctions in renal epithelial cells**

Epithelial cells are uniquely equipped with tight junctions, which not only maintain epithelial polarity but also function as a barrier to prevent free diffusion of solutes. Trans-epithelial electrical resistance (TER) is used as a measure to determine the tightness of cell–cell contact mediated by the tight junctions (20, 21). Electrical cell-substrate impedance sensing (ECIS) technology was used to continually monitor TER in MDCK and NCX1-KD cells. Cells were plated in wells fitted with gold electrodes. A constant alternating current was applied between the electrodes. The increase in resistance to the current due to the attachment of cells and formation of junctions was recorded. TER values were normalized to the initial value, and the graph was plotted as described previously (22). The TER increased gradually over time and reached a plateau. Even after the TER attained a plateau, it was monitored for several more hours. MDCK cells showed normalized peak TER of 23.1 at 8.2 h. In contrast, the peak TER attained by NCX1-KD cells was only 12.9, and it required 11.8 h. Thus, normalized TER of NCX1-KD cells over the entire time range was significantly lower than for MDCK cells (*p* < 0.0001), indicating that the junctions are compromised in NCX1-KD cells (Fig. 4A). A calcium-switch assay was used to study synchronized de novo junction formation as described previously (23). MDCK cells assembled junctions very rapidly and attained peak TER that was 5.6-fold higher than baseline within 3.2 h. The peak TER attained by NCX1-KD cells was only 4.1-fold higher than baseline and required 10.8 h (Fig. 4B). These data further support the conclusion that NCX1-KD cells do not achieve high TER in comparison with MDCK cells and that the time taken to reach maximum TER is longer in NCX1-KD cells compared with MDCK cells.

Interestingly, MDCK cells treated with KB-R7943 to inhibit NCX1 activity did not show appreciable change in
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The adherens junctions also appeared to be affected in NCX1-KD cells, whereas desmosomal structures looked similar to MDCK cells, indicating that reduced NCX1 affects the ultrastructure of cell–cell junctions.

**NCX1 associates with and stabilizes E-cadherin on the cell surface**

E-cadherin is a key junctional protein that is required for the formation of tight and adherens junctions (24). Therefore, we tested the expression level of E-cadherin in NCX1-KD cells. These cells not only showed decreased total E-cadherin protein but also showed 50% reduction in membrane-localized E-cadherin determined in a cell surface biotinylation assay (Fig. 5A).

The intracellular trafficking of E-cadherin is a dynamic process that regulates E-cadherin function (25). Therefore, we examined E-cadherin exocytosis and endocytosis in NCX1-KD cells. The amount of E-cadherin protein that reached and was retained at the cell membrane within 15 min in NCX1-KD cells was not significantly different from that in MDCK cells (Fig. 5B). There was a striking difference in the amount of E-cadherin endocytosed in NCX1-KD cells. Almost 100% of E-cadherin was endocytosed in NCX1-KD cells, whereas only 3% E-cadherin was internalized in MDCK cells in 15 min (Fig. 5B).

These data indicate that E-cadherin in NCX1-KD cells is destabilized and probably undergoes rapid internalization soon after it reaches the cell surface.

Therefore, the membrane stability of E-cadherin was evaluated by a detergent extraction assay. In this assay, proteins that are tightly linked to the submembranous actin cytoskeleton are not easily extracted by detergent treatment and are mostly retained in the pellet fraction. E-cadherin in MDCK cells is tightly linked to the actin cytoskeleton. Thus, only 26% of E-cadherin was Triton X-100-soluble in these cells and detected in the supernatant fraction (5 in Fig. 5C). In comparison, 64% of E-cadherin was Triton X-100 soluble in NCX1-KD cells (Fig. 5C), suggesting that E-cadherin is not stably anchored to the plasma membrane. NCX1-KD cells also showed a concomitant reduction in the E-cadherin level in the pellet fraction (P). β-Actin remained in the pellet fraction following detergent extraction in both MDCK and NCX1-KD cells, indicating that NCX1 knockdown does not affect the structure of the actin cytoskeleton.

The association between NCX1 and E-cadherin was tested, because NCX1 appears to regulate E-cadherin expression. A reciprocal co-immunoprecipitation analysis revealed that E-cadherin and β-catenin co-immunoprecipitates with anti-NCX1 antibody, and NCX1 was pulled down by anti-E-cadherin antibody (Fig. 5D). Furthermore, immunofluorescence microscopy showed that NCX1 and E-cadherin co-localized on the cell surface (Fig. 5E). These data suggest that NCX1 associates with E-cadherin in MDCK cells.

To determine whether the interaction between NCX1 and E-cadherin is dependent on NCX1 ion transport activity, MDCK cells were treated with 10 μM KB-R7943 (NCX1 inhibitor). This is the half-maximal concentration required for inhibition of NCX1 activity accompanied by increase in intracellular calcium in MDCK cells (13, 26). Furthermore, we showed earlier that 10 μM KB-R7943 induced cell migration in these...
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Figure 5. NCX1 interacts with E-cadherin and regulates its membrane expression independent of NCX1 ion transport activity. A, immunoblot representing total and membrane E-cadherin expression along with total β-catenin levels. β-Actin was used as a loading control. B, representative immunoblots of exocytosis (quenched with or without NHS-acetate) and endocytosis (reduced or non-reduced) of E-cadherin in MDCK and NCX1-KD cells. C, immunoblots of E-cadherin and β-actin in soluble (S) and pellet (P) fractions in MDCK and NCX1-KD cells following detergent extraction. D, co-immunoprecipitation assay showing E-cadherin, β-catenin, and NCX1 protein in IgG, anti-NCX1, and anti-E-cadherin immunoprecipitates (IP). E, E-cadherin and β-catenin antibodies were simultaneously added for the detection of both proteins on the same blot. F, immunofluorescence images showing co-localization of NCX1 and E-cadherin in MDCK cells. Bar, 10 μm. G, lysates of MDCK cells treated with DMSO or KB-R7943 were immunoprecipitated using anti-E-cadherin antibody and immunoblotted for NCX1 and E-cadherin. Note that E-cadherin co-immunoprecipitated with NCX1 in NCX1-inhibited cells. H, representative immunoblots showing E-cadherin and β-actin in detergent-soluble (S) and pellet (P) fractions of MDCK monolayers treated with DMSO (control) or KB-R7943.

cells with no toxicity (13). The quantity of NCX1 immunoprecipitated by anti-E-cadherin antibody in MDCK cells treated with KB-R7943 was similar to that in DMSO-treated MDCK control cells, indicating that NCX1 inhibition does not affect their interaction (Fig. 5F, top). The level of E-cadherin also remained unaltered following treatment with KB-R7943 (Fig. 5F, bottom). The membrane stability of E-cadherin in cells treated with KB-R7943 was also tested. The percentages of E-cadherin in supernatant (S) and pellet (P) fraction in control and KB-R7943 treated cells remained similar (Fig. 5G), signifying no effect of NCX1 functional inhibition on E-cadherin membrane localization. Taken together, these data suggest that NCX1–E-cadherin complex formation stabilizes E-cadherin on the membrane independent of NCX1 ion transport function.

NCX1 suppresses β-catenin transcriptional activity

β-Catenin is an interaction partner of E-cadherin that anchors it to the membrane via its interaction with other catenins and cytoskeletal proteins; in addition, it also functions as a transcriptional activator (27). When E-cadherin is destabilized from the membrane, β-catenin is internalized and translocated into the nucleus, where it binds with transcription factors lymphoid enhancer-binding factor-1 (LEF) and T cell factor 3 (TCF), regulating transcription of target genes (28, 29). Because NCX1 knockdown destabilizes E-cadherin and reduces its membrane expression, nuclear translocation of β-catenin was tested. Immunostaining of β-catenin in NCX1-KD cells displayed the presence of β-catenin in the cytoplasm and the nucleus, whereas a similar localization of β-catenin was not observed in MDCK cells (Fig. 6A).

To determine whether the nuclear localization of β-catenin in NCX1-KD cells is associated with enhanced β-catenin transcriptional activity, a β-catenin reporter assay was performed. In this reporter assay, the luciferase activity produced by the binding of β-catenin to TCF/LEF sites upstream of luciferase reporter (TOPflash) and a scrambled promoter coupled to Renilla luciferase activity. Renilla luciferase vector was co-transfected
as an internal control to determine the transfection efficiency. The luciferase activity produced by the activation of the TOPflash reporter after deducting the FOPflash background luciferase activity following normalization with Renilla luciferase activity was 2-fold higher in NCX1-KD cells compared with MDCK cells (Fig. 6B). Furthermore, targets of β-catenin transcrip-tional activity were evaluated to determine whether the downstream signaling pathway was activated. NCX1-KD cells showed 2.6-fold higher cyclin D1 transcript level and 2.5-fold greater Axin2 transcript level compared with MDCK cells (Fig. 6C). These data indicated that the β-catenin signaling pathway was induced in NCX1-KD cells.

XAV-939, a Wnt-β-catenin inhibitor was utilized to test the role of β-catenin signaling in NCX1-KD cells (30). Treatment with 2 μM XAV-939 for 96 h resulted in the generation of a greater proportion of polarized cysts (66.5%) in NCX1-KD cells plated on Matrigel (Fig. 6D), suggesting that the non-polarized cysts in NCX1-KD required β-catenin signaling.

**NCX1 knockdown increases mesenchymal markers**

As described above, the morphology of NCX1-KD cells was fibroblastic with non-compact tight junctions. Moreover, the epithelial marker E-cadherin was down-regulated. Therefore, the levels of mesenchymal markers were tested to determine whether NCX1 knockdown induced EMT in MDCK cells. Mesenchymal markers α-smooth muscle actin (α-sma), fibronectin, and N-cadherin were elevated (Fig. 7, A and B). NCX1-KD cells also exhibited an increase in the phosphorylation and nuclear localization of nuclear factor-κB (NF-κB) (Fig. 7B), which governs fibronectin expression during EMT (31, 32). Fibronectin and NF-κB are known to be regulated by β-catenin. Therefore, we evaluated whether these two proteins are regulated by β-catenin signaling in NCX1-KD cells. Fibronectin protein was greatly reduced in NCX1-KD cells treated with XAV-939, whereas phosphorylated NF-κB levels did not show appreciable change (Fig. 7C). Thus, these data indicate that knockdown of NCX1 induces EMT in MDCK cells associated with β-catenin transcriptional activation.

Anchorage-independent growth is a hallmark feature of transformed epithelial cells. NCX1-KD cells formed colonies in soft agar independent of anchorage, unlike MDCK cells (Fig. 8, A and B), suggesting that NCX1-KD cells have gained tumorigenic potential. Taken together, these data demonstrate that NCX1 knockdown reduces E-cadherin membrane stability and increases β-catenin transcriptional activity, leading to EMT and increased tumorigenic potential.

**Discussion**

This study demonstrated that NCX1 transcript and protein levels are reduced in renal cancers. Knockdown of NCX1 in MDCK cells induced fibroblastic morphology and prevented lumen formation in 3D cultures. In addition, NCX1-KD cells had reduced TER and increased permeability with concomitant increase in intercellular junctional space. These cells were capable of anchorage-independent growth and showed increased proliferation. Moreover, NCX1-KD cells displayed elevated mesenchymal markers and reduced epithelial marker E-cadherin. Mechanistically, we demonstrate that NCX1 inter-acts with E-cadherin and stabilizes it on the plasma membrane. Destabilization of E-cadherin is associated with activation of β-catenin signaling pathway in cells with NCX1 knockdown. Thus, NCX1-KD cells acquired characteristics of mesenchymal cells, signifying that knockdown of NCX1 induces EMT.

Evidence demonstrating the involvement of Ca2+ regulators, such as transient receptor potential (TRP) channels and PMCA, in cancer progression has been gained recently. Some Ca2+ regulators are altered in tumors. Reductions in TRPC3
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breast/ovarian cancer, TRPC6 in glioma, TRPM7 in ovarian cancers, and PMCA in breast cancers were observed (33–36). Functional inhibition or knockdown of these proteins reduced cell proliferation, migration, and metastasis (37–39), thus confirming the role of these proteins in cancer induction. A recent report showed that reduction in NCX1 protein in penile carcinoma was associated with increased proliferation and reduced apoptosis (40). NCX1 gene expression was reduced in chemoresistant variants of an ovarian cancer cell line, suggesting a role for NCX1 in sensitization to chemotherapy (41). Reduction in NCX1 transcript level was also observed in breast cancer cells undergoing transforming growth factor-β-induced EMT (42). These correlative data lend support to our observation that NCX1 is reduced in renal cancers. This is the first study showing the effect of knockdown of NCX1 on epithelial characteristics.

Multiple studies have highlighted the role of calcium in the regulation of cellular functions, such as proliferation, apoptosis, and migration (43–45). Because NCX1 is responsible for calcium extrusion, it was unsurprising that NCX1-KD cells showed 1.5-fold higher intracellular calcium (data not shown). Thus, it is likely that some altered cellular functions observed in NCX1-KD cells are caused by an increase in intracellular calcium. For example, elevation in intracellular calcium may be responsible for high proliferation in NCX1-KD cells. Likewise, we showed previously that functional inhibition of NCX1 with KB-R7943 induced migration (13). On the contrary, increase in intracellular calcium by treatment with KB-R7943 did not affect the TER in MDCK cells, indicating that the pump function of NCX1 is probably not involved in tight junction formation (Fig. 4B). This is in agreement with various studies showing that changes in intracellular calcium do not modulate tight junction permeability, although extracellular calcium is essential for tight junction formation and maintenance (20, 23). The ion transport function of NCX1 is well characterized, but this is the first report highlighting the role of NCX1 independent of its ion transport function.

An important phenomenon during organ development, especially in kidney, breast, and prostate organogenesis, is the reorganization of epithelial cells to form spheroids with lumen and tubular networks (46). The development of carcinomas in these organs is associated with disrupted lumen polarity and the formation of multiple lumens with eventual filling of the luminal space characteristic of EMT (47). Unlike parental MDCK, NCX1-KD cells formed bigger non-polarized cysts or cysts with multiple lumens, associated with the loss of apicobasal polarity. Moreover, these cysts were larger, probably due to an increase in rate of proliferation, because enhanced proliferation rate has been shown to increase the size of the cysts with p120 knockdown (48). Cysts with multiple lumens and progressive enlargement of the cyst are features observed in the initial pre-invasive stage of carcinoma associated with loss of cellular function (47, 49, 50). Moreover, NCX1-KD cells also exhibited anchorage-independent growth, confirming the carcinogenic potential of these cells.

NCX1 interacts with proteins, such as calmodulin, phosphatases, PP2A, and Na,K-ATPase β-subunit (51–53), that have been implicated in regulation of cancer progression (54–56). In this study, we show that NCX1 co-localizes and co-immunoprecipitates with E-cadherin and regulates E-cadherin cell surface expression. It remains to be determined whether NCX1 directly interacts with E-cadherin or via another protein, such as β-catenin. Inhibition of NCX1 activity neither prevented NCX1–E-cadherin association nor affected E-cadherin membrane stability, demonstrating that NCX1 regulation of E-cadherin is independent of its ion transport activity.

The dissociation of adherens junctions by loss or destabilization of E-cadherin can promote β-catenin nuclear localization (27, 57). Once inside the nucleus, β-catenin can bind with LEF/TCF and act as a transcription factor, ultimately resulting in activation of the Wnt–β-catenin signaling pathway, which can induce EMT (28, 58–62). We observed nuclear localization and activation of β-catenin signaling in NCX1-KD cells. Blocking this signaling induced epithelial polarization in 3D cultures and suppressed fibronectin accumulation, highlighting the role of β-catenin in the induction of EMT in NCX1-KD cells (Fig. 9). Similar to NCX1-KD cells, RCC and Wilms tumor samples also showed induction of cyclin D1 transcript levels (data not shown), indicative of β-catenin transcriptional activation. Activation of Wnt–β-catenin pathway is a common occurrence in both RCC and Wilms tumor, although it is not associated with mutations in β-catenin (63, 64). It is tempting to speculate that loss of NCX1 regulates E-cadherin and promotes the activation of β-catenin signaling in these tumors.

During kidney development, cycles of MET and EMT are synchronized by the expression of specific transcription factors, such as WT-1, Egr-1, Pax2, and Pax8. These proteins are highly expressed in metanephric mesenchymal cells. During generation of functional renal tubules by differentiation of metanephric mesenchymal cells to epithelial cells, the expression of
these transcription factors is down-regulated (65). Interestingly, aberrant expression of these proteins has been observed both in Wilms tumor (where mesenchymal cells do not differentiate into epithelial cells during development) and in RCC (where epithelial cells are transformed to mesenchymal cells) (66–68). Conversely, NCX1 expression is reduced in both Wilms tumor and RCC, indicating that NCX1 expression is directly proportional to epithelial phenotype. Egr-1 is known to regulate NCX1 in the heart (69). Thus, it is likely that Egr-1 may also regulate NCX1 during kidney development. How NCX1 is down-regulated in RCC remains to be determined. Taken together, our data suggest that NCX1 may function as a potential tumor suppressor. Further investigation on the role of NCX1 in tumorigenesis is currently in progress in the laboratory.

**Experimental procedures**

### Cell lines and tumor samples

Madin-Darby canine kidney (MDCK) from American Type Culture Collection (Manassas, VA), as described previously (70), and Lenti-XTM HEK-293T cells (632180) from Clontech (Palo Alto, CA) were cultured in DMEM with 10% fetal bovine serum, 2 mM/L L-glutamine, 25 units/ml penicillin, and 25 μg/ml streptomycin.

For the generation of MDCK cells with stable knockdown of NCX1, lentiviral transduction was used for genomic integration of the shRNA expression cassette. Two distinct shRNAs targeted against canine NCX1 (sequence 1, AATGGAGGGAAGGCCAATGA; sequence 2, AAGTCGAGGTTCAAGCTTG) and scrambled shRNA (AACGTACGCGGAATCTCAGG) were cloned into the lentiviral vector pLKO.1 puro, a gift from Bob Weinberg (Addgene plasmid 8453) (71). Lentiviral particles were generated by co-transfection of this plasmid with pMDL/g/pRRE (Addgene plasmid 12251), pMD2.G (Addgene plasmid 12259), and pRSV-Rev (Addgene plasmid 12253) in HEK-293T cells. The three packaging plasmids were a gift from Didier Trono (72). The lentiviral particles were used for the transduction of MDCK cells. Two days post-transduction, cells were cultured in the presence of 10 μg/ml puromycin for selection pressure and maintained with 2 μg/ml puromycin. No puromycin was added in cells plated for experiments.

Wilms tumor and RCC tissues (n = 6 each) were obtained along with their matched normal tissues from the Nemours BioBank and the Helen F. Graham Cancer Center of Christiana Care Hospital, respectively, under an Institutional Review Board-approved protocol with informed patient consent.

### Antibodies and reagents

Anti-NCX1 monoclonal antibody (clone: C2C12, ab2869) from Abcam® (Cambridge, MA) was used in a 1:1000 dilution. Anti-β-actin (clone: AC-74, A5316) and anti-E-cadherin (clone: DECMA-1, U3254) monoclonal antibodies from Sigma-Aldrich were used in 1:5000 and 1:1000 dilution, respectively. Anti-N-cadherin (clone: 32, 610920), anti-fibronectin (clone: 10, 610077), anti-phospho-NF-κb p65 (clone: 20, 610868), and anti-β-catenin (clone: 14, 610153) from BD Biosciences were used in 1:2000, 1:1000, 1:1000, and 1:2000 dilution, respectively. Anti-PMCA1 (bs-4978R) polyclonal antibody from BIOSS (Woburn, MA) was used in 1:1000 dilutions. Horseradish peroxidase-conjugated secondary monoclonal antibodies against mouse IgG (catalog no. 7076), rabbit IgG (catalog no. 7054), and mouse IgG light chain (clone: D3V2A, 58802) obtained from Cell Signaling Technology (Lexington, KY) were used in 1:1000 dilution. XAV939 was purchased from Cayman Chemical (Ann Arbor, MI).

### Immunoblot analysis and co-immunoprecipitation

The cells were lysed with buffer containing 95 mM NaCl, 25 mM Tris, pH 7.4, 0.5 mM EDTA, 2% SDS, 1 mM PMSF, 5 mg/ml mixture of protease inhibitor (PI). Similarly, tumor samples were lysophilized with liquid nitrogen and lysed in buffer containing 1% Triton X-100, 100 mM Tris-Cl, 200 mM NaCl, 0.2% SDS, 5 mM EDTA, 100 μg/ml PI mixture. After quantitation with a Bio-Rad protein assay kit, lysates containing 100 μg of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were blocked in 5% milk in TBS with 0.1% Tween 20 (TBST) for 1 h and incubated with primary antibodies diluted in 5% BSA in TBST overnight at 4 °C. After washes with TBST, the blots were incubated with secondary antibodies in 5% milk in TBST for 1 h and developed with a chemiluminescent Western Lightning™ system according to the manufacturer’s recommendations, ECL or ECL Prime (GE Healthcare). TINA version 2.0 software (Straubenhardt, Germany) was used for quantification of immunoblots.

For co-immunoprecipitation, MDCK cells were either untreated or treated with DMSO (0.01%) or 10 μM KB-7943 for 8 h wherever indicated. Cell lysates corresponding to 1 mg of total protein were incubated overnight with Protein G Magagarose (GE Biosciences, Pittsburgh, PA) precoupled with antibodies for 4 h. The beads were washed, and proteins bound to the beads were separated on SDS-PAGE and immunoblotted as described above.

### Quantitative RT-PCR analysis

RNA was obtained by TRIzol® extraction method, and cDNA was synthesized by the iScript™ cDNA synthesis kit (Bio-Rad) as per the manufacturer’s instructions. The cDNA was amplified using the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). The following primers were used for tumors: (a) NCX1 (forward, GACCTCGGTCTCTAGCAGCAT; reverse, TTGTCTGTCACTTCTCTGGTT) and (b) tubulin (forward, CTGTGATGAGCTGCTCAGGGTGG; reverse, GTTGGCCAGGCCGGTGTCCAG). The following primers were used for MDCK cells: (a) cyclin D1 (forward, AGCGACGGAAGTGCGAGG; reverse, CACACTCTGTCGACAGGCG); (b) Axin2 (forward, GGCAAATGCGTGGCAGTGAT); (c) GAPDH (forward, CCCTGACACCAACTGCTT; reverse, GTCTTCTGCTTGGCATGTAT). Quantitative PCR was performed in a 384-well plate on a 7900HT fast real-time PCR system (Applied Biosystems). Samples were assayed in triplicate, and transcript levels were calculated by relative quantification, normalizing the samples to the endogenous control GAPDH or tubulin. Graphs represent the
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average of the relative mean expression level (relative quantification value) of three different experiments, and the error bars represent the S.E. (GraphPad Software Inc., La Jolla, CA).

Immunofluorescence and confocal microscopy

100,000 cells were plated on coverslips. After 24 h, cells were fixed with either ice-cold methanol at −20 °C or 4% paraformaldehyde in PBS at room temperature. The coverslips were blocked with 1% BSA in PBS for 30 min, followed by incubation with primary antibody for 2 h and fluorescence-tagged secondary antibody for 1 h. The coverslips were washed with PBS, incubated with TO-PRO-3 iodide (Thermo Fisher Scientific, Carlsbad, CA) for 10 min, and mounted onto the glass slides with ProLong gold antifade reagent (Thermo Fisher Scientific). Images were captured using a TCS SP5 laser-scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL) with a 63/1.4 numeric aperture oil immersion objective lens.

Cell length measurement

Average cell length was calculated from a minimum of 20 cells, randomly selected, per field of view. A scale bar drawn across the length of the cell in LSM software (Leica Microsystems) provided cell length measurement, as described previously (70).

3D cultures and imaging

MDCK and NCX1-KD cells were grown in 3D MatrigelTM, as demonstrated earlier (70). 8-Chambered LAB-TEK II dishes from Nalgene Nunc (Rochester, NY) were coated with growth factor-reduced 2% MatrigelTM from BD Biosciences. 15,000 cells/ml were plated in these coated chambers in DMEM and incubated at 37 °C. Medium was replaced after 48 h, and the cysts were fixed after 96 h. XAV-939 was added when cells were plated on Matrigel.

Cells grown in 3D cultures were blocked with PFS (0.7% fish skin gelatin, 0.1% saponin (Sigma)) for 30 min, followed by overnight incubation with anti-β-catenin antibody diluted in PFS. After washes with PFS, the cysts were incubated with anti-mouse Alexa-488 and phalloidin-conjugated with Alexa-546 for 2 h, followed by incubation with TO-PRO-3 iodide for 30 min. The cyst images were captured using a TCS SP5 confocal microscope. At least 100 cysts were randomly selected per experiment, the numbers of polarized and non-polarized cysts were counted, and the cyst size was measured using LSM software as described above.

Cell proliferation assay

BrdU cell proliferation assay kit from Biovision (Milpitas, CA) was used to compare the rate of proliferation between MDCK and NCX1 KD cells. 10,000 cells were plated per well in a 96-well plate. After 24 h, the cells were treated with 20 μl of BrdU at 37 °C for 4 h. The cells were then fixed with fixing/denaturing solution and stained with 1× BrdU detection antibody solution for 1 h. After two washes with 1× wash buffer, 1× anti-mouse HRP-linked antibody solution was added and incubated for 1 h. Tetramethylbenzidine substrate was added and absorbance was measured at 650 nm, and then stop solution was added and absorbance at 450 nm was measured.

TER measurement assay

2 × 10⁵ cells were plated in 200 μl of medium in each well of ECIS electrode arrays (8W10E), with active gold electrodes in the base, and allowed to incubate for 20 h. The resistance levels were continually monitored at 500 Hz using ECIS model 1600R (Applied BioPhysics, Troy, NY), and TER values were normalized with respect to the initial TER value, which was considered 1, as described previously (22).

Ca2+ switch assay

MDCK cells were trypsinized and plated on 8W10E ECIS array plate. The cells were allowed to attach for 1 h in DMEM containing 1.8 mM Ca2+. Then the inserts were gently rinsed in SMEM (minimum essential medium for suspension culture) (Invitrogen) containing < 5 μM Ca2+ and 5% dialyzed FBS and then incubated with calcium-free SMEM overnight at 37 °C. After 16 h, SMEM was removed, complete DMEM was added to the cells, and the changes in the TER were continuously monitored. KB-R9743 or DMSO was added with complete DMEM wherever indicated.

Solute permeability assay

The permeability assay was performed as described previously (73, 74). 200,000 cells were plated onto 12-mm polyester transwell inserts with a pore of 0.4-μm size (Corning Inc.) and incubated for 48 h. After 48 h, 0.5 μM TAMRA (Sigma-Aldrich) was added to the apical chamber, and 50 μl of medium from the basal chamber was sampled every 15 and 30 min for 3 h. 10 μl of the medium was removed from the apical chamber at the start and end of the experiment. The collected samples were pipetted onto a black 96-well plate and measured (excitation at 560 nm and emission at 590 nm) in a microplate reader (PerkinElmer Life Sciences).

Transmission electron microscopy

Transmission electron microscopy was performed as described previously with certain modifications (75). Cells were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 h at room temperature. The cells were washed with 0.1 M sodium cacodylate buffer and embedded in 4% low-melting point agarose. The agarose was cut into 1–2 mm³ cubes, washed again with 0.1 M sodium cacodylate buffer, and fixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h. Further, the cubes were dehydrated in ascending acetone solution (25, 50, 75, 95, and 100%) after a 15-min incubation in each solution. The cubes were then infiltrated with Embed-812 resin in a decreasing ratio of acetone to fresh Embed-812 resin (from 1 part Embed-812 resin:3 parts acetone to 100% Embed-812 resin) every 1 h. The agarose cubes were embedded into labeled BEEM capsules with fresh Embed-812 resin and polymerized for 1 h at 65 °C. The samples were sectioned and imaged in a Zeiss Libra 120 transmission electron microscope (Thornwood, NY).

Anchorage-independent growth assay

1.5 ml of DMEM with 0.5% noble agar was plated in 6-well dishes and air-dried. Cells were trypsinized, and 10,000 cells
were resuspended per ml of DMEM with 0.3% noble agar. 1.5 ml of these cells were then plated on air-dried bottom layer and incubated for 3 weeks at 37 °C, with the addition of 200 μl of DMEM two times per week. After 14 days, the cells were stained with 200 μl of nitro blue tetrazolium chloride (Sigma-Aldrich) solution and incubated at 37 °C overnight. The colonies were imaged using a Geliance 600 imaging system and analyzed with GeneTools™ software (both from PerkinElmer Life Sciences).

Cell surface biotinylation assay

Subconfluent cells were labeled with 0.5 μg/ml membrane-impermeable EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in triethanolamine (TEA) buffer (150 mM NaCl, 10 mM TEA, pH 9, 1 mM CaCl₂, 1 mM MgCl₂) on ice. After 20 min, the cell surfaces were quenched with ammonium chloride (50 mM NH₄Cl in PBS, 0.1 mM CaCl₂, 1 mM MgCl₂) and lysed in 300 μl of lysis buffer (150 mM NaCl, 20 mM Tris, pH 8, 5 mM EDTA, 1% Triton X-100, 0.1% BSA, 1 mM PMSF, 1 mg/ml PI mixture). The lysates were incubated with 30 μl of Ultraplink streptavidin beads (Thermo Scientific) at 4 °C, and after 16 h, the beads were washed and immunoblotted.

Exocytosis biotinylation assay

The exocytosis biotinylation assay was performed as described previously (76). Cells grown in 6-well dishes were chilled on ice and quenched with 2 mg/ml Sulfo-NHS-acetate (Pierce) dissolved in PBS-CM for 20 min to saturate NHS-reactive sites on the cell surface. A control dish was chilled on ice but not quenched. After quenching for 20 min, cells were incubated at 37 °C for 0 or 15 min to allow protein trafficking. At this point, cells were chilled on ice and labeled with 1.5 mg/ml Sulfo-NHS-biotin. Following biotinylation, cells were lysed and incubated at 4 °C with streptavidin beads overnight, and the proteins bound to the beads were resolved by SDS-PAGE and immunoblotted for E-cadherin.

Endocytosis biotinylation assay

EZ-Link Sulfo-NHS-SS-Biotin (Pierce) was used to biotinylate cell surface proteins at 4 °C, as described previously (77). Two control dishes were retained on ice, whereas experimental dishes were incubated at 37 °C for the indicated times and then returned to ice. One control dish was not reduced and served as input (100%). The remaining dishes were incubated with reducing solution (250 mM of glutathione, 10 mM NaCl, 250 mM NaOH, 10% fetal bovine serum) to remove the biotin on the cell surface. The free sulfhydryl groups of biotin were quenched in 5 mg/ml iodoacetamide (Sigma-Aldrich) plus 5% BSA in PBS. The cells were lysed and incubated with Ultraplink Streptavidin beads overnight. Following wash, the biotinylated proteins bound to the beads were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-cadherin.

Detergent extraction assay

Confluent monolayer of cells were incubated with 200 μl of extraction buffer (50 mM NaCl, 10 mM PIPES, pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100, 300 mM sucrose, 1 mM PMSF, 100 units/ml DNase) for 10 min at 4 °C. The lysates were centrifuged at 4 °C for 30 min at 14,000 rpm in a microcentrifuge. The supernatant was removed, and 200 μl of sample buffer was added to it. The pellet was resuspended in 200 μl of the extraction buffer and sonicated, and 200 μl of sample buffer was added. Equal volumes of supernatant and pellet fraction were resolved on a gel and immunoblotted as described above. Wherever indicated, MDCK cells were treated with DMSO (0.1%) or 10 μM KB-R7943 for 8 h before detergent extraction.

β-Catenin transcriptional activity

100,000 MDCK and NCX1-KD cells were co-transfected with 1 μg of either reporter plasmid M50 Super 8× TOPFlash (Addgene plasmid 12456), containing luciferase reporter of β-catenin-mediated transcriptional activation or control plasmid M51 Super 8× FOPFlash (Addgene plasmid 12457) containing luciferase reporter with mutated TCF/LEF binding sites, a gift from Randall Moon (78) and pRL-SV40-Renilla luciferase expression construct (Promega, Madison, WI) (GenBank™ vector accession number AF025845). After 48 h, the transfected cells were lysed with passive lysis buffer, and the supernatant was assayed with the Dual-Glo luciferase assay kit (Promega) for firefly and Renilla luciferase activity in a VIC-TOR X multilabel plate reader (PerkinElmer Life Sciences). The luciferase activity produced by the binding of β-catenin to the LEF/TCF binding sites (TOPflash) was subtracted from the luciferase activity produced by the scrambled promoter (FOPflash), and the transfection efficiency was normalized to Renilla luciferase activity. The mean luciferase activity from three independent experiments was calculated, and the -fold change was compared between MDCK and NCX1-KD cells.

Statistical analysis

The results from three independent experiments are represented as means ± S.E. in graphs generated with GraphPad Prism software. The statistical significance between experimental groups was calculated by paired or unpaired Student’s t test, and p < 0.05 was considered statistically significant.

Author contributions—S. L. B. conducted the experiments and analyzed and interpreted the results. A. G. generated material for experiments and provided intellectual insights for the project development. N. J. P. provided the tumor samples and helped in analysis and interpretation of the data. S. P. B. conceived the idea for the project, secured funding, interpreted results, and wrote the paper with S. L. B.

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References

1. Little, M. H., and McMahon, A. P. (2012) Mammalian kidney development: principles, progress, and projections. Cold Spring Harb. Perspect. Biol. 10.1101/cshperspect.a008300
2. Hohenstein, P., Pritchard-Jones, K., and Charlton, J. (2015) The yin and yang of kidney development and Wilms’ tumors. Genes Dev. 29, 467–482
NCX1 knockdown induces EMT

3. Schedl, A. (2007) Renal abnormalities and their developmental origin. *Nat. Rev. Genet.* 8, 791–802

4. Dumanski, Y. V., Kudriashov, A. G., Vasilenko, I. V., Kondratyuk, R. B., Gulkov, Y. K., and Cyrillichystiakov, R. S. (2013) Markers of epithelial-mesenchymal transition in renal cell carcinoma. *Exp. Oncol.* 35, 325–327

5. Harada, K., Miyake, H., Kusuda, Y., and Fujisawa, M. (2012) Expression of epithelial-mesenchymal transition markers in renal cell carcinoma: impact on prognostic outcomes in patients undergoing radical nephrectomy. *BJU Int.* 110, E1131–E1137

6. Tun, H. W., Marlow, L. A., von Roemeling, C. A., Cooper, S. J., Kreinest, P., Wu, K., Luxon, B. A., Sinha, M., Anastasiadis, P. Z., and Copland, J. A. (2010) Pathway signature and cellular differentiation in clear cell renal cell carcinoma. *PLoS One* 5, e10696

7. Srigley, J. R., and Delahunt, B. (2009) Uncommon and recently described renal carcinomas. *Mod. Pathol.* 22, S2–S23

8. van der Hagen, E. A., van Loon, E. P., Verkaart, S., Latta, F., Bindels, R. J., and Hoenderop, J. G. (2015) The Na+/Ca2+ exchanger 1 (NCX1) variant 3 as the major extrusion system in renal distal tubular transcellular Ca2+ transport. *Nephron* 131, 145–152

9. Li, Z., Matsuoaka, S., Hryshko, L. V., Nicoll, D. A., Bersohn, M. M., Burke, E. P., Lifron, R. P., and Philipson, K. D. (1994) Cloning of the NCX2 isoform of the plasma membrane Na+-Ca2+ exchanger. *J. Biol. Chem.* 269, 17434–17439

10. Lytton, J. (2007) Na+/Ca2+ exchangers: three mammalian gene families control Ca2+ transport. *Biochem. J.* 406, 365–382

11. Nicoll, D. A., Quednau, B. D., Qui, Z., Xia, Y. R., Lusis, A. J., and Philipson, K. D. (1996) Cloning of a third mammalian Na+-Ca2+ exchanger, NCX3. *J. Biol. Chem.* 271, 24914–24921

12. Hilge, M., Aelen, J., and Vuister, G. W. (2006) Ca2+ regulation in the Na+/Ca2+ exchanger involves two markedly different Ca2+ sensors. *Mol. Cell* 22, 15–25

13. Balasubramaniam, S. L., Gopakrishnapillai, A., Gangadharan, V., Duncan, R. L., and Barwe, S. P. (2015) Sodium-calculator 1 regulates epithelial cell migration via calcium-dependent extracellular signal-regulated kinase signaling. *J. Biol. Chem.* 290, 12463–12473

14. Chambers, K. F., Pearson, J. F., Pellacani, D., Aziz, N., Guzvic, K., Klein, C. A., and Lang, S. H. (2011) Stromal upregulation of lateral epithelial adhesions: gene expression analysis of signalling pathways in prostate epithelium. *J. Biomed. Sci.* 18, 45

15. Yusenko, M. V., Zubakov, D., and Kovacs, G. (2009) Gene expression profiling of chromophobe renal cell carcinomas and renal oncocytomas by Affymetrix GeneChip using pooled and individual tumours. *Int. J. Biol. Sci.* 5, 517–527

16. Yusenko, M. V., Kuiper, R. P., Boethe, T., Ljungberg, B., van Kessel, A. G., and Kovacs, G. (2009) High-resolution DNA copy number and gene expression analyses distinguish chromophobe renal cell carcinomas and renal oncocytomas. *BMC Cancer* 9, 152

17. Cutcliffe, C., Kersey, D., Huang, C. C., Zeng, Y., Walterhouse, D., Perlman, E. J., and Renal Tumor Committee of the Children’s Oncology Group (2005) Clear cell sarcoma of the kidney: up-regulation of neural markers with activation of the sonic hedgehog and Akt pathways. *Clin. Cancer Res.* 11, 7986–7994

18. Jones, J., Otu, H., Spentzos, D., Sill, I., Benecken, W. D., Fellman, C., Gu, X., Joseph, M., Pantuck, A. J., Jonas, D., and Libermann, T. A. (2005) Gene signatures of progression and metastasis in renal cell carcinoma. *J. Urol.* 173, 609–618

19. Muñoz, J. I., Drigo, S. A., Barros-Filho, M. C., Marchi, F. A., Scapulariometano, C., Sousa, G. S., Guimarães, G. C., Trindade Filho, J. C., Lopes, A., Arruda, M. A., and Rogatto, S. R. (2015) Down-regulation of SCLCA1 as a putative apoptosis evasion mechanism by modulation of calcium levels in penile carcinoma. *J. Urol.* 194, 245–251

20. Januchowski, R., Sawierucha, P., Rucinski, M., Andrzejewska, M., Wojtowicz, K., Nowicki, M., and Zabel, M. (2014) Drug transporter expression profiling in chemoresistant variants of the A2780 ovarian cancer cell line. *Biochem. Pharmacol.* 88, 477–485

21. Mahdi, S. H., Cheng, H. L., and Feng, R. (2015) The effect of TGF-β-induced epithelial-mesenchymal transition on the expression of intracellular calcium-handling proteins in T47D and MCF-7 human breast cancer cells. *Arch. Biochem. Biophys.* 583, 1–26

22. Stock, C., and Schwab, A. (2015) Ion channels and transporters in metas-tasis. *Biochim. Biophys. Acta* 1848, 2638–2646
44. Yang, S. L., Cao, Q., Zhou, K. C., Feng, Y. J., and Wang, Y. Z. (2009) Transient receptor potential channel C3 contributes to the progression of human ovarian cancer. Oncogene 28, 1320–1328

45. Monteith, G. R., Davis, F. M., and Roberts-Thomson, S. J. (2012) Calcium channels and pumps in cancer: changes and consequences. J. Biol. Chem. 287, 31666–31673

46. Zegers, M. M., O’Brien, L. E., Yu, W., Datta, A., and Mostov, E. K. (2003) Epithelial polarity and tubulogenesis in vitro. Trends Cell Biol. 13, 169–176

47. Debnath, J., and Brugge, J. S. (2005) Modelling glandular epithelial cancers in three-dimensional cultures. Nat. Rev. Cancer 5, 675–688

48. Marciano, D. K., Brekman, P. R., Lee, C. Z., Spivak, N., Eastburn, D. J., Bryant, D. M., Beaudoin, G. M., 3rd, Hofmann, I., Mostov, K. E., and Reichardt, L. F. (2011) p120 catenin is required for normal renal tubulogenesis and glomerulogenesis. Development 138, 2099–2109

49. Hanaoka, K., and Guggino, W. B. (2000) cAMP regulates cell proliferation and cyst formation in autosomal polycystic kidney disease cells. J. Am. Soc. Nephrol. 11, 1179–1187

50. Sullivan, L. P., Wallace, D. P., and Grantham, J. J. (1998) Epithelial transport in polycystic kidney disease. Physiol. Rev. 78, 1165–1191

51. Chou, A. C., Ju, Y. T., and Pan, C. Y. (2015) Calmodulin interacts with the sodium/calcium exchanger NCX1 to regulate activity. PLoS One 10, e013856

52. Schulze, D. H., Muqbal, M., Lederer, W. J., and Ruknudin, A. M. (2003) Sodium/calcium exchanger (NCX1) macromolecular complex. J. Biol. Chem. 278, 28849–28855

53. Balasubramaniam, S. L., Gopalakrishnapillai, A., and Barve, S. P. (2015) Ion dependence of Na-K-ATPase-mediated epithelial cell adhesion and migration. Am. J. Physiol. Cell Physiol. 309, C437–C441

54. Hais, W. N., and Lazo, J. S. (1986) Calmodulin: a potential target for cancer chemotherapeutic agents. J. Clin. Oncol. 4, 994–1012

55. Perrotti, D., and Neviani, P. (2013) Protein phosphatase 2A: a target for cancer therapy. Lancet Oncol. 14, e229–e238

56. Inge, L. J., Rajasekaran, S. A., Yoshimoto, K., Mischel, P. S., McBride, W., Hait, W. N., and Lazo, J. S. (2008) Evidence for a potential tumor suppressor gene (WT1) in renal cell carcinoma. J. Am. Soc. Nephrol. 19, 1135–1142

57. Barve, S. P., Sky, A., McSpadden, R., Huyhn, T. P., Langhans, S. A., Inge, L. J., and Rajasekaran, A. K. (2012) Na-K-ATPase β-subunit cis homo-oligomerization is necessary for epithelial lumen formation in mammalian cells. J. Cell Sci. 125, 5711–5720

58. Stewart, S. A., Dykshoorn, D. M., Palliser, D., Mizuno, H., Yu, E. Y., An, D. S., Sabatini, D. M., Chen, I. S., Hahn, W. C., Sharp, P. A., Weinberg, R. A., and Novina, C. D. (2003) Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 9, 493–501

59. Barve, S. P., Skay, A., Mandel, R. J., Nguyen, M., Trono, D., and Naldini, L. (1998) A third-generation lentivirus vector with a conditional packaging system. J. Virol. 72, 8463–8471

60. Phillips, B. E., Cancel, L., Tarbell, J. M., and Antonetti, D. A. (2008) Occludin independently regulates permeability under hydrostastic pressure and cell division in retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 49, 2568–2576

61. Bollinger, M. T., Ramzheker, A., Waldschmidt, H. V., Larsen, S. D., Bewley, M. C., Flanagan, J. M., and Antonetti, D. A. (2016) Occludin S471 phosphorylation contributes to epithelial monolayer maturation. Mol. Cell. Biol. 36, 2051–2066

62. Modla, S., Caplan, J. L., Czyzewski, K. J., and Lee, J. Y. (2015) Localization of fluorescently tagged protein to plasmodesmata by confocal light and electron microscopy. Methods Mol. Biol. 1217, 121–133

63. Carmosino, M., Rizzo, F., Procino, G., Basco, D., Valenti, G., Forbush, B., Schaeren-Wieters, N., Caplan, M. J., and Svelto, M. (2010) MAL/VIP17, a new player in the regulation of NKCC2 in the kidney. Mol. Biol. Cell 21, 3985–3997

64. Zhang, X., Yang, M., Shi, H., Hu, J., Wang, Y., Sun, Z., and Xu, S. (2017) Reduced E-cadherin facilitates renal cell carcinoma progression by WNT/β-catenin signaling activation. Oncotarget 8, 19566–19576

65. Lechner, M. S., and Dressler, G. R. (1997) The molecular basis of embryonic kidney development. Mech. Dev. 62, 105–120

66. Campbell, C. E., Kuriyan, N. P., Rackley, R. R., Caulfield, M. J., Tubbs, R., Finke, J., and Williams, B. R. (1998) Constitutive expression of the Wilms tumor suppressor gene (WT1) in renal cell carcinoma. Int. J. Cancer 78, 182–188

67. Ozcan, A., de la Roza, G., Ro, J. Y., Shen, S. S., and Tsung, L. D. (2012) PAX2 and PAX8 expression in primary and metastatic renal tumors: a comprehensive comparison. Arch. Pathol. Lab. Med. 136, 1541–1551

68. Hansson, M. L., Behmer, S., Ceder, R., Mohammad, S., Preta, G., Grafov, R. C., Fadeel, B., and Wallberg, A. E. (2012) MAML1 acts cooperatively with EGR1 to activate EGR1-regulated promoters: implications for nephrogenesis and the development of renal cancer. PLoS One 7, e66001

69. Pacini, L., Saffredini, S., Ponti, D., Coppini, R., Frati, G., Ragona, G., Cerba, E., and Calogero, A. (2013) Altered calcium regulation in isolated cardiomyocytes from Egr-1 knock-out mice. Can. J. Physiol. Pharmacol. 91, 1135–1142

70. Bollinger, M. T., Ramzheker, A., Waldschmidt, H. V., Larsen, S. D., Bewley, M. C., Flanagan, J. M., and Antonetti, D. A. (2016) Occludin S471 phosphorylation contributes to epithelial monolayer maturation. Mol. Cell. Biol. 36, 2051–2066

71. Modla, S., Caplan, J. L., Czyzewski, K. J., and Lee, J. Y. (2015) Localization of fluorescently tagged protein to plasmodesmata by confocal light and electron microscopy. Methods Mol. Biol. 1217, 121–133

72. Carmosino, M., Rizzo, F., Procino, G., Basco, D., Valenti, G., Forbush, B., Schaeren-Wieters, N., Caplan, M. J., and Svelto, M. (2010) MAL/VIP17, a new player in the regulation of NKCC2 in the kidney. Mol. Biol. Cell 21, 3985–3997

73. Anilkumar, G., Rajasekaran, S. A., Wang, S., Hankinson, O., Bander, N. H., and Rajasekaran, A. K. (2003) Prostate-specific membrane antigen association with filament A modulates its internalization and NAA1Dase activity. Cancer Res. 63, 2645–2648

74. Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H., and Moon, R. T. (2003) Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr. Biol. 13, 680–685

75. Vestweber, D., Kemler, R., and Eklom, P. (1985) Cell-adhesion molecule uromorulin during kidney development. Dev. Biol. 112, 213–221

76. Natho, A., and Takeichi, M. (1988) Cell binding function of E-cadherin is regulated by the cytoplasmic domain. EMBO J. 7, 3679–3684

77. Rhodes, D. R., Yu, J., Shander, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A., and Chinnaiyan, A. M. (2004) ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia 6, 1–6