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Detailed methods

Yeast two hybrid

A yeast two hybrid (Y2H) screen was performed by Hybrigenics (Hybrigenics Services, S.A.S., Paris, France (http://www.hybrigenics-services.com)) on a mouse total kidney library with the first 133 amino acids of rat NCC as the bait fragment.

Immunoprecipitation

MDCK type I cells with tetracycline inducible FLAG-tagged NCC expression were grown until confluent on polyester supports and induced for 18 hrs with tetracycline (1μg/μl). Cells were lysed in 20 mm Tris, 150 mm NaCl, 1% Triton-X, 0.01% SDS, pH7.4, containing protease inhibitors Leupeptin and Pefabloc and phosphatase inhibitor mixture tablets (PhosSTOP, Roche Diagnostics). NCC was immunoprecipitated (IP) from the cleared lysate using a rabbit anti-FLAG antibody. Similar IPs without antibody or use of an AQP1 antibody served as a negative control.

Immunofluorescence staining and quantification

6-7 acute kidney slices (280 μm) from 3 mice (3 independent experiments) were prepared and incubated with isoproterenol 100 nmol/L or vehicle as described before. After stimulation, slices were fixed by immersion in 3% paraformaldehyde (PFA) prepared in 0.1 mol/L phosphate buffer (pH 7.4, 300 mOsm). After washing in similar buffer without PFA, whole slices were mounted on an organic support, frozen in liquid propane and sectioned (4 μm) to proceed with immunofluorescence staining as previously described [1].

For the quantification of fluorescence intensity, at least two sets of 4 consecutive sections (4 μm) per slice (280 μm) were stained with the following primary antibodies tI1, pT35I1, tNCC and pT53NCC. The sections were imaged (1 image / 4 μm section) using a Leica SP8 inverse STED 3X confocal microscope equipped with a 20X multi-immersion objective (HC PL APO CS2). Within each image, all DCTs that could be identified in all four consecutive sections were selected for quantification (4-15 DCTs / image). The mean fluorescence intensity of the whole DCT was quantified using ImageJ and divided by the mean intensity of a non-stained area of the same image to correct for background differences. The intensities of all DCTs within one image were averaged to obtain one value per image per antibody. Similarly, the intensity of all

images coming from the same 280 μm slice stained with the same antibody were averaged to obtain one value per slice which is represented in figure 6.

Isolated perfused mouse kidney

Isolated mouse kidney perfusion was performed at 37°C in a small animal perfusion system (Hugo Sachs Elektronik, Germany) as previously described [2]. To reduce the scattering of NCC activation between animals, the Renin-Angiotensin-Aldosterone System (RAAS) was suppressed 2 days prior to the experiment by feeding age and weight matched male WT and I1 deficient mice with 8% NaCl diet. In all experiments, kidneys were perfused for 40 minutes with control buffer before isoproterenol was added to a final concentration of 100 nmol/L. Kidneys were further perfused for another 40 minutes and finally snap frozen in liquid nitrogen for western blot analysis.

Electron Microscopy

Kidney slices were cut with a vibratome as described before and incubated in control buffer for 0, 30 or 60 min. Afterwards, slices were fixed overnight in 3% PFA and 1% glutaraldehyde in 0.1 mol/L phosphate buffer pH 7.3, 300 mOsm. The slices were then rinsed in 0.1 mol/L phosphate buffer before post-fixation in 1% osmium tetroxide in phosphate buffer for 2h at room temperature. Following dehydration through a graded series of ethanol, the slices were infiltrated and embedded in Epoxy embedding medium (Fluka, Seelze, Germany) overnight at room temperature. Thin sections (70 – 75 nm) were cut on a Leica EM FCS ultramicrotome and collected onto 100 mesh copper grids. Sections were stained with uranyl acetate 30 min and Reynold's lead citrate 10 min. Grids were analyzed on a Philips CM 100 (Eindhoven, The Netherlands) at 80 kV.

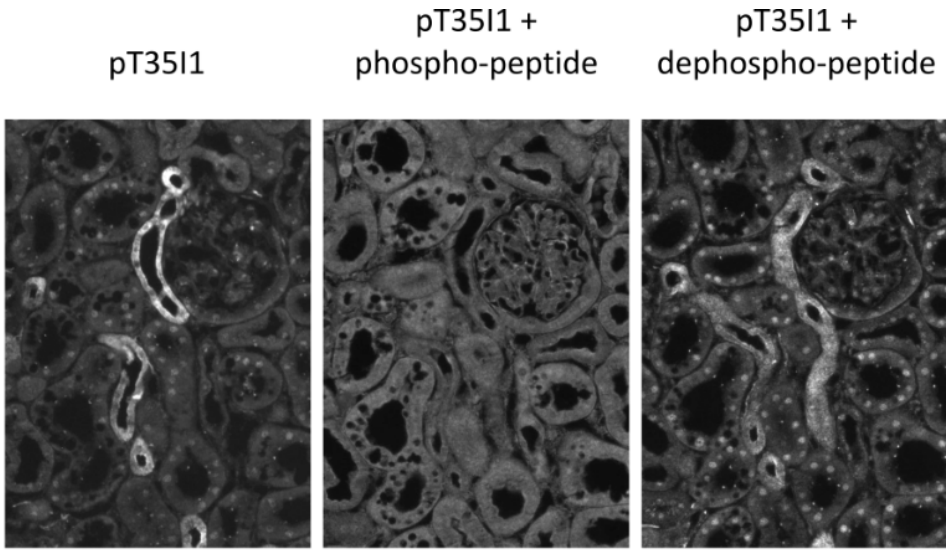
Automated DCT isolation

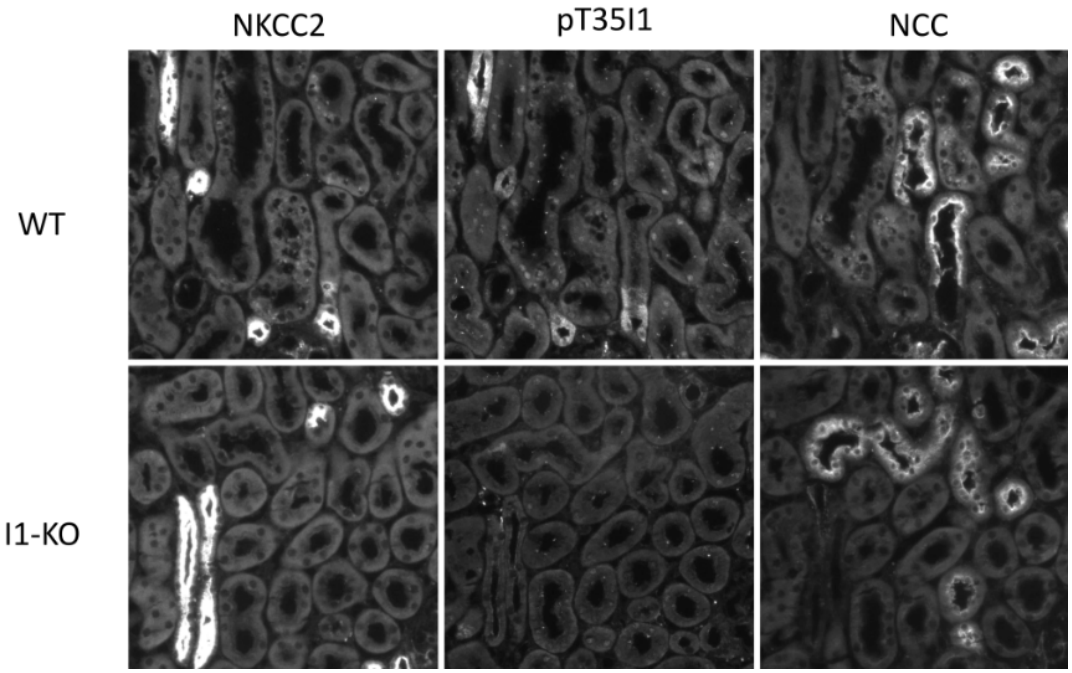
To sort single renal DCT1 fragments, mice expressing EGFP under the control of the parvalbumin promoter (PV-EGFP) [3] were anesthetized with isoflurane (Attane, Piramal, India) and perfused through the heart first with 10 ml of cold PBS and then with 10 ml of digestion solution (1mg/ml collagenase (Worthington Biochemical Corporation, Lakewood, NJ08701, USA); 1mg/ml hyaluronidase and 0.1mg/ml DNaseI prepared in ice-cold KREBS (in mmol/L): 130 NaCl, 10 HEPES, 3 KCl, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.8 $MgSO_4$, 10 glucose, pH 7.3). The renal cortexes from both kidneys were dissected under a stereomicroscope. Samples were finely minced and digested in 20 ml of fresh

digestion solution at 30°C for 17 minutes. The tubular digest was first filtered through 250- and 212-µm nylon sieves. The flow-through was then filtered with a 100-µm and a 40-µm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). The tubules retained by the 40-µm cell strainer were diluted with ice-cold Krebs to a total volume of 50 ml. All sortings were performed with a large particle sorter (BioSorter) instrument (Union Biometrica, Somerville, MA). The following instrument settings were used: delay 10 mS, width 6.5 mS, PMT 350 volt, sample cup pressure 8.5 psi, pre-analysis chamber pressure 4.8-5.4 psi. The sample fluid pressure was set to maintain a sort frequency of 10-20 events/s. The mixer speed was 50%. Sorted tubules were collected directly into ice-cold KREBS and centrifuged at 800g for 4 minutes.

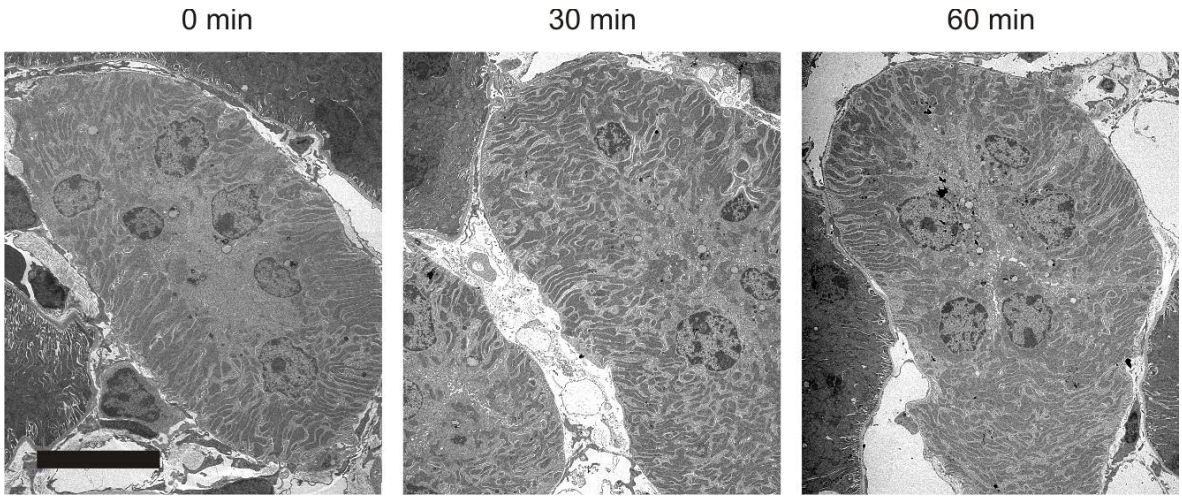
After sorting, a suspension of 800 DCT fragments was treated with either vehicle (DMSO) or 10 µmol/L FSK + 100 µmol/L IBMX, or 20 µmol/L H-89 or 1-10 µmol/L of PKI 14-22 amide, myristoylated or a combination of FSK/IBMX plus one of the PKA inhibitors. Treatment lasted 20 min and was performed at room temperature. After treatment, samples were shortly centrifuged, the supernatant was removed and tubuli were resuspended in Laemli buffer and further processed for immunoblot as described elsewhere[4].

Supplementary figure 1: Assessment of the specificity of the pT35I1 antibody by immunohistochemistry. **A**: Consecutive sections of kidneys from WT animals stained with pT53I1 antibody (left panel), the same antibody previously incubated with the phospho-peptide NH2-CRRRP(pT)PATL-CONH2 (middle panel) or pre-incubated with the dephospho-peptide NH2-CRRRPTPATL-CONH2 (right panel). **B**: Consecutive sections of kidneys from WT animals (upper panel of three images) or I1-KO mice (lower panel of three images) stained with NKCC2, NCC and pT35I1 antibodies.

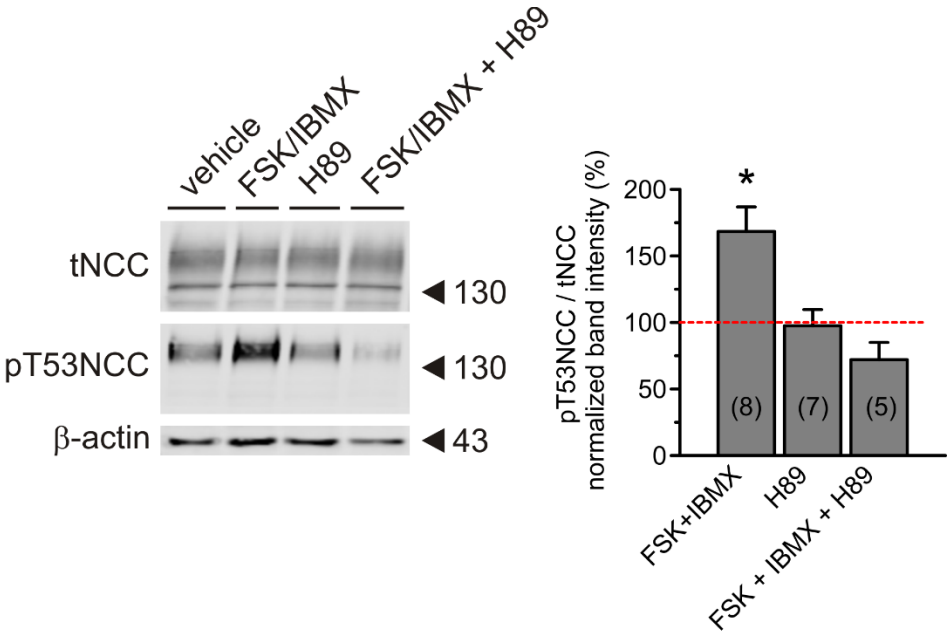




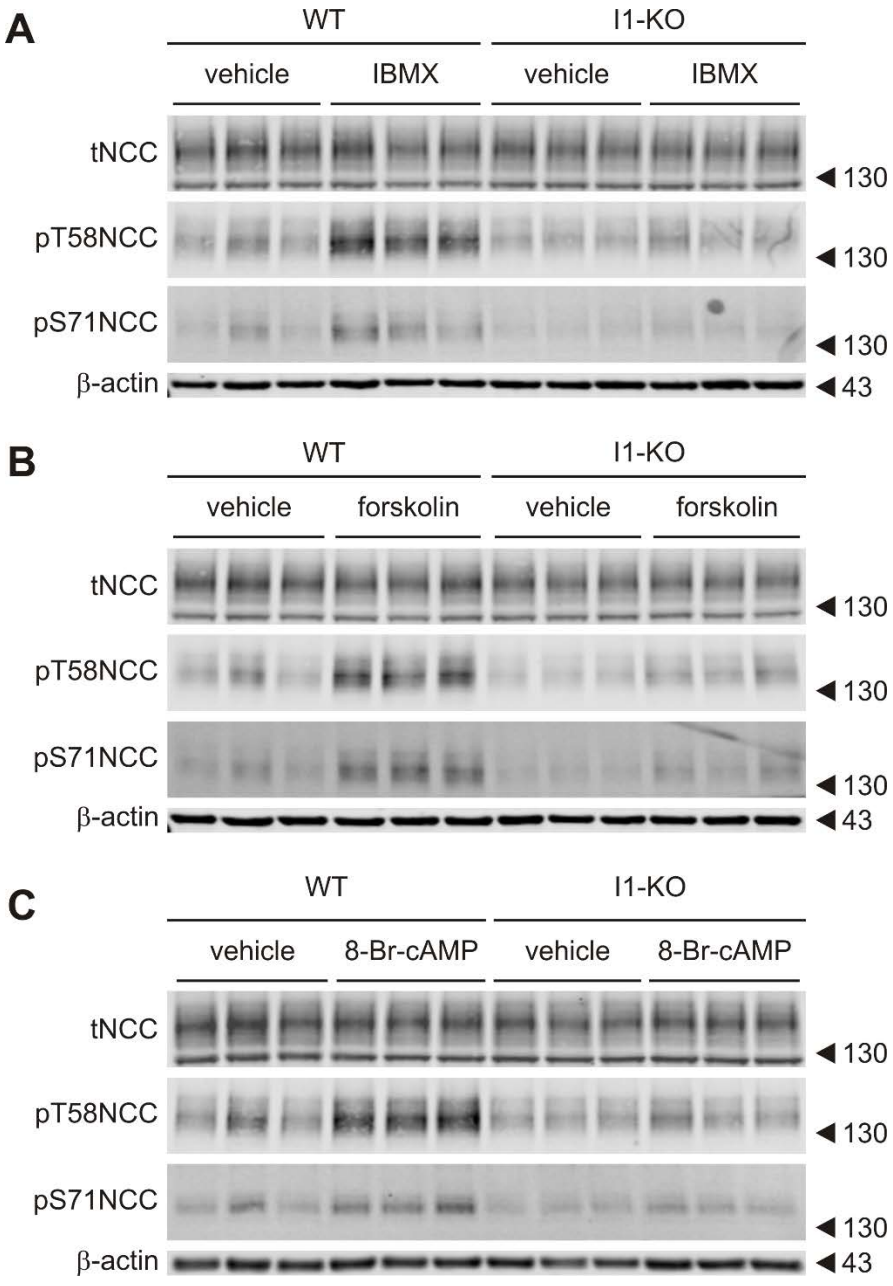
Supplementary figure 2: Assessment of DCT integrity in kidney slices from WT mice immediately after slicing (left panel) or incubated in control solution during 30 min (middle panel) and 60 min (right panel). Scale bar 10 μm.



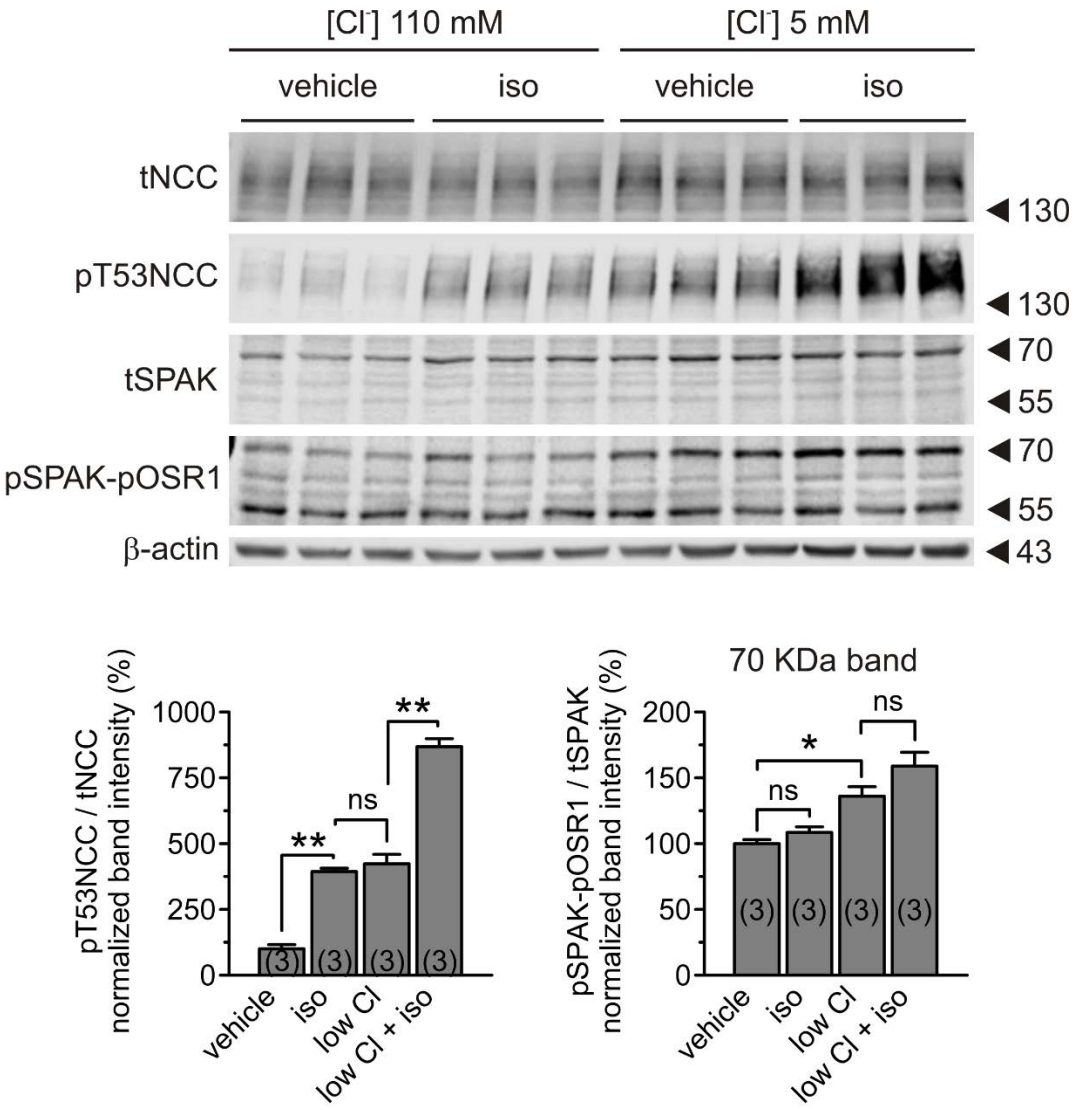
Supplementary figure 3: Inhibition of cAMP-dependent phosphorylation of NCC by the PKA inhibitor H-89. Left panel represents a typical immunoblot of isolated DCTs treated with 1) vehicle, 2) 10 μmol/L FSK + 100 μmol/L IBMX, 3) 20 μmol/L H-89 and 4) FSK+IBMX+H-89 in the same concentrations previously used. Each lane corresponds to 400 DCT fragments. On the right panel, a densitometric analysis of the pT53NCC/tNCC from 5-8 independent experiments ("n" in brackets) normalized to control vehicle group (red line) is represented. *p<0.05 compared to control vehicle condition and assessed by one-way ANOVA followed by Tukey's multiple comparisons test.



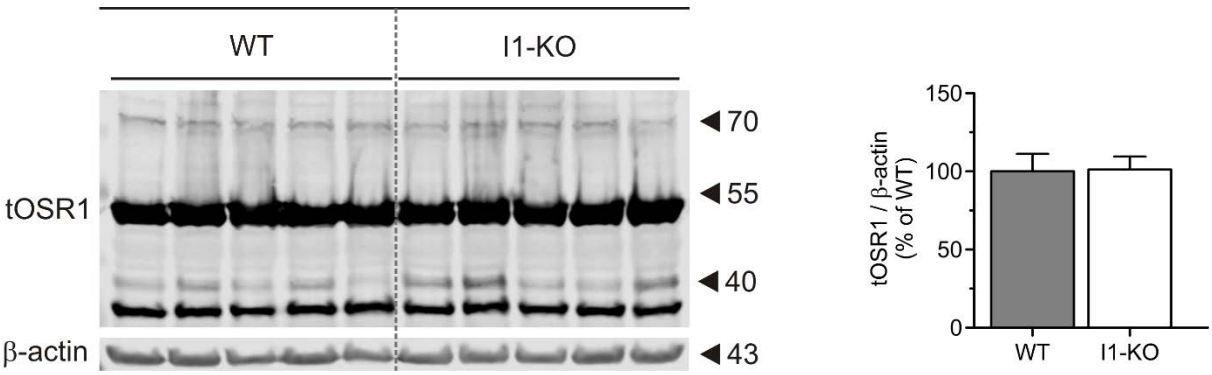
Supplementary figure 4: Stimulation of NCC phosphorylation at T58 and S71 with cAMP elevating agents. Representative immunoblots showing the effect of (A) IBMX (100 μmol/L), (B) forskolin (10 μmol/L) and (C) 8-Br-cAMP (10 μmol/L) on NCC phosphorylation at position T58 and S71 in WT and I1-KO kidney slices. The densitometric quantification of the immunoblots is available in supplementary table 1.



Supplementary figure 5: Effect of low Cl^- on the stimulation of NCC and SPAK-OSR1 phosphorylation by isoproterenol in kidney slices of WT mouse. Graph represents the densitometric analysis of the phosphorylation of NCC at T53 (left) or the 70 KDa band of pSPAK-pOSR1 immunoblot (right) normalized to control vehicle. * p<0.05, **p<0.01, ns: non-significant assessed by one-way ANOVA followed by Tukey's multiple comparisons test. n= 3 slices (in brackets) from 1 mouse.



Supplementary figure 6: Expression of OSR1 in WT and I1-KO mice. The graph represents the densitometric quantification of tOSR1/β-actin in all bands shown normalized to WT.



Supplementary table 1: Densitometric quantification of Supplementary Figure 4

Summary					
FSK stimulation					
	mean	**SEM**	**n**	**Two-way-ANOVA + Tukey's multiple comparisson**	**adjusted p value**
WT_vehicle_pT58NCC	1	0.094367	3	WT vehicle vs FSK	<0.0001
WT_FSK_pT58NCC	2.499423	0.081614	3	I1-Ko vehicle vs FSK	0.0029
I-1_KO_vehicle_pT58NCC	1	0.070559	3	WT FSK vs I1-KO FSK	0.0051
I-1_KO_FSK_pT58NCC	1.784053	0.147475	3		
WT_vehicle_pS71NCC	1	0.073505	3	WT vehicle vs FSK	<0.0001
WT_FSK_pS71NCC	2.691473	0.169197	3	I1-KO vehicle vs FSK	0.006
I-1_KO_vehicle_pS71NCC	1	0.072473	3	WT FSK vs I1-KO FSK	0.0039
I-1_KO_FSK_pS71NCC	1.815317	0.136574	3		
IBMX stimulation					
	mean	**SEM**	**n**	**Two-way-ANOVA + Tukey's multiple comparisson**	**adjusted p value**
WT_vehicle_pT58NCC	1	0.083918	3	WT vehicle vs IBMX	<0.0001
WT_IBMX_pT58NCC	2.290356	0.080265	3	I1-KO vehicle vs IBMX	0.1023
I-1_KO_vehicle_pT58NCC	1	0.047617	3	WT IBMX vs I1-KO IBMX	<0.0001
I-1_KO_IBMX_pT58NCC	1.300646	0.095564	3		
WT_vehicle_pS71NCC	1	0.159223	3	WT vehicle vs IBMX	0.0039
WT_IBMX_pS71NCC	1.936363	0.184994	3	I1-KO vehicle vs IBMX	0.1004
I-1_KO_vehicle_pS71NCC	1	0.068808	3	WT IBMX vs I1-KO IBMX	0.1493
I-1_KO_IBMX_pS71NCC	1.494105	0.047443	3		
8-Br-cAMP stimulation					
	mean	**SEM**	**n**	**Two-way-ANOVA + Tukey's multiple comparisson**	**adjusted p value**
WT_vehicle_pT58NCC	0.991639	0.107951	3	WT vehicle vs 8-Br-cAMP	0.0008
WT_8-Br-cAMP_pT58NCC	1.970833	0.118414	3	I1-KO vehicle vs 8-Br-cAMP	0.9156
I-1_KO_vehicle_pT58NCC	1.051336	0.034929	3	WT 8-Br-cAMP vs I1-KO 8-Br-cAMP	0.0025
I-1_KO_8-Br-cAMP_pT58NCC	1.146948	0.132396	3		
WT_vehicle_pS71NCC	1.272917	0.166387	3	WT vehicle vs 8-Br-cAMP	0.0008
WT_8-Br-cAMP_pS71NCC	2.193591	0.214361	3	I1-KO vehicle vs 8-Br-cAMP	0.9156
I-1_KO_vehicle_pS71NCC	1.506866	0.130078	3	WT 8-Br-cAMP vs I1-KO 8-Br-cAMP	0.0025
I-1_KO_8-Br-cAMP_pS71NCC	1.769712	0.217122	3		

Supplementary table 2: Densitometric quantification of pT58NCC shown in figure 3D of the main manuscript

	mean	SEM	n
pT58NCC wt vehicle	1.00	0.09	3
pT58NCC WT Calyc_A	2.01	0.20	3
pT58NCC I1-KO vehicle	1.00	0.13	3
pT58NCC I1-KO Calyc_A	2.18	0.42	3

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4. Penton D, Czogalla J, Wengi A, Himmerkus N, Loffing-Cueni D, Carrel M, Rajaram RD, Staub O, Bleich M, Schweda F, Loffing J: Extracellular K(+) rapidly controls NaCl cotransporter phosphorylation in the native distal convoluted tubule by Cl(-) -dependent and independent mechanisms. *J. Physiol.* 594: 6319–6331, 2016