Protein Phosphatase 1 Inhibitor–1 Mediates the cAMP-Dependent Stimulation of the Renal NaCl Cotransporter

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Abstract: Background: A number of cAMP-elevating hormones stimulate phosphorylation (and hence activity) of the NaCl cotransporter (NCC) in the distal convoluted tubule (DCT). Evidence suggests that protein phosphatase 1 (PP1) and other protein phosphatases modulate NCC phosphorylation, but little is known about PP1’s role and the mechanism regulating its function in the DCT. Methods: We used ex vivo mouse kidney preparations to test whether a DCT-enriched inhibitor of PP1, protein phosphatase 1 inhibitor–1 (I1), mediates cAMP’s effects on NCC, and conducted yeast two-hybrid and coimmunoprecipitation experiments in NCC-expressing MDCK cells to explore protein interactions. Results: Treating isolated DCTs with forskolin and IBMX increased NCC phosphorylation via a protein kinase A (PKA)–dependent pathway. Ex vivo incubation of mouse kidney slices with isoproterenol, norepinephrine, and parathyroid hormone similarly increased NCC phosphorylation. The cAMP-induced stimulation of NCC phosphorylation strongly correlated with the phosphorylation of I1 at its PKA consensus phosphorylation site (a threonine residue in position 35). We also found an interaction between NCC and the I1-target PP1. Moreover, PP1 dephosphorylated NCC in vitro, and the PP1 inhibitor calyculin A increased NCC phosphorylation. Studies in kidney slices and isolated perfused kidneys of control and I1-KO mice demonstrated that I1 participates in the cAMP-induced stimulation of NCC. Conclusions: Our data suggest a complete signal transduction pathway by which cAMP increases NCC phosphorylation via a PKA-dependent phosphorylation of I1 and subsequent inhibition of PP1. This pathway might be relevant for the physiologic regulation of renal sodium handling by cAMP-elevating hormones, and may contribute to salt-sensitive hypertension in patients with endocrine disorders or sympathetic hyperactivity.

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David Penton¹,², Sandra Moser¹, Agnieszka Wengi¹, Jan Czogalla¹,², Lena Lindtoft Rosenbaek³,⁴, Fritz Rigendinger¹, Nourdine Faresse¹,², Joana R. Martins¹,², Robert A. Fenton³, Dominique Loffing-Cueni¹, Johannes Loffing¹,²

¹Institute of Anatomy, University of Zurich, Switzerland; ²Swiss National Centre for Competence in Research “Kidney control of homeostasis”; ³Department of Biomedicine, Aarhus University, Denmark; Department of Neuroscience, University of Copenhagen

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Protein phosphatase 1 inhibitor 1 mediates the cAMP-dependent stimulation of the renal NaCl cotransporter

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¹Institute of Anatomy, University of Zurich, Switzerland; ²Swiss National Centre for Competence in Research “Kidney control of homeostasis”; ³Department of Biomedicine, Aarhus University, Denmark; Department of Neuroscience, University of Copenhagen

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Corresponding author:
Johannes Loffing
University of Zurich, Institute of Anatomy
Winterthurerstrasse 190, CH-8057 Zurich
Switzerland
Phone: +41 (0) 44 635 53 20
Fax: +41 (0) 44 635 57 02
Email: johannes.loffing@anatom.uzh.ch
Significance statement:

The thiazide-sensitive NaCl cotransporter (NCC) in the distal convoluted tubule (DCT) is critical for the renal control of ion homeostasis and blood pressure. NCC phosphorylation, and hence activity, is increased by cAMP-elevating stimuli, including β-adrenergic agonists and PTH. We tested the hypothesis that the inhibitor 1 (I1) of protein phosphatase 1 (PP1) mediates the effects of cAMP-elevating hormones on NCC. Using several in vitro and ex vivo approaches, we propose a novel signaling pathway in which a PKA-dependent phosphorylation of I1 inhibits the PP1-dependent dephosphorylation of NCC. This novel pathway may contribute to the physiological regulation of NCC and the development of arterial hypertension in the context of abnormal hormonal stimulation.
Abstract

Background: The NaCl cotransporter (NCC) in the distal convoluted tubule (DCT) is critical for renal Na⁺ reabsorption and blood pressure control. A number of cAMP-elevating hormones stimulate NCC phosphorylation, and hence activity.

Methods: Using a variety of experiments on mouse ex vivo kidney preparations, we tested the hypothesis that the DCT-enriched protein phosphatase 1 inhibitor 1 (I1) mediates the effects of cAMP on NCC.

Results: Treatment of isolated DCTs with forskolin and IBMX increased the phosphorylation of NCC via a protein kinase A (PKA)-dependent pathway. Likewise, ex vivo incubation of mouse kidney slices with isoproterenol, norepinephrine and PTH increased NCC phosphorylation. The cAMP-induced stimulation of NCC phosphorylation strongly correlated with the phosphorylation of I1 at its PKA consensus site (T35). Yeast two-hybrid and co-immunoprecipitation experiments in NCC-expressing MDCK cells indicated an interaction between NCC and the I1-target PP1. Moreover, PP1 dephosphorylated NCC in vitro and the PP1 inhibitor calyculin A increased NCC phosphorylation. Studies on kidney slices and isolated perfused kidneys of control and I1-KO mice demonstrated that I1 participates to the cAMP-induced stimulation of NCC.

Conclusion: Our data suggests a complete signal transduction pathway by which cAMP increases NCC phosphorylation via a PKA-dependent phosphorylation of I1 and subsequent inhibition of PP1. This pathway might be relevant for the physiological regulation or renal Na⁺ handling by cAMP-elevating hormones and may contribute to salt-sensitive hypertension in patients with endocrine disorders or sympathetic hyperactivity.

Keywords:
NaCl cotransporter, β-adrenergic stimulation, PKA, protein phosphatase 1, protein phosphatase 1 inhibitor 1
Introduction

The thiazide-sensitive NaCl cotransporter (NCC) in the renal distal convoluted tubule (DCT) is crucial for the fine-tuning of renal sodium (Na⁺) reabsorption and hence for the control of blood pressure. NCC and the DCT are also critically involved in the renal control of potassium (K⁺), magnesium (Mg²⁺), calcium (Ca²⁺) and acid/base homeostasis. The crucial role of NCC is evidenced by genetic diseases in which loss of function mutations of NCC cause Gitelman syndrome featuring hypokalemic alkalosis, hypomagnesemia, hypocalciuria and lowered arterial blood pressure. Conversely, enhanced NCC activity due to mutations in its regulating kinases, namely the WNK kinases WNK1 and WNK4, cause Familial Hyperkalemic Hypertension (FHH) with hypermagnesemia and hypercalciuria. Moreover, mutations in ubiquitin-ligase complex proteins such as kelch-like-3 (KLHL3) and cullin-3 (CUL3), which control WNK4 stability, are also causative of FHH. The WNK kinases control NCC activity via the STE20/SPS1-related proline- and alanine-rich kinase (SPAK) and the oxidative stress-response kinase 1 (OSR1). SPAK and OSR1 directly phosphorylate NCC at several serine and threonine residues located within the N-terminal tail of the cotransporter.

The activity of the WNK-SPAK kinase pathway and NCC is regulated by various factors including the renin-angiotensin-aldosterone (RAAS) system. Although the DCT expresses the cognate receptors for angiotensin II and aldosterone, recent work suggests that the effect of these hormones on NCC is indirectly mediated via changes in plasma K⁺ concentration ([K⁺]). Plasma [K⁺] is proposed to modulate WNK4 activity through changes in DCT membrane voltage and intracellular Cl⁻ concentration. Other NCC stimulators such as the β-adrenergic agonist isoproterenol as well as the parathyroid hormone (PTH) are thought to mediate their effects via intracellular cAMP. Recently, the cAMP-dependent protein kinase (PKA) was implicated in the regulation of WNK4, suggesting that cAMP may also act via the WNK/SPAK kinase pathway. However, these studies were mainly performed in heterologous expression systems and it remained unclear whether this and/or additional pathways contribute to the cAMP-dependent regulation of NCC in the native DCT. Some studies suggested that the kinase OSR1 and the extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein kinase (MAPK) are also involved in the activation of NCC by catecholamines and PTH, respectively.
Despite the progress on the elucidation of the role and regulation of the WNK/SPAK/OSR1 kinase pathway, little is known about the phosphatases that counterbalance the action of these kinases. As yet, three protein phosphatases (PP) were suggested to modulate NCC phosphorylation; PP1, PP3 (calcineurin) and PP4. In *Xenopus laevis* oocytes, heterologous co-expression of NCC with PP4 lowered NCC phosphorylation\(^\text{17}\). Likewise, pharmacological inhibition of PP1 with calyculin A \(^\text{18}\) and of PP3 with tacrolimus \(^\text{19,20}\) increased NCC phosphorylation in various experimental settings. The stimulatory effect of PP3 inhibition on NCC may have important clinical implications. In fact, a common side effect of calcineurin-inhibitor treatment is renal sodium retention and arterial hypertension, which correlates with an enhanced urinary excretion of phosphorylated NCC\(^\text{18,21}\). Nevertheless, the physiological role of the different phosphatases in the DCT and of the underlying mechanism regulating their function are unclear.

Interestingly, both the catalytic activity and the substrate-specificity of phosphatases are often modulated by the interaction with specific regulatory subunits. We recently found that the endogenous inhibitor 1 (I1) of protein phosphatase 1 is highly expressed in the DCT with strong effects on NCC phosphorylation and arterial blood pressure\(^\text{22}\). I1 is a small 171 amino acid cytosolic protein encoded by the Ppp1r1a gene\(^\text{23}\). It is expressed in many organs including the brain, skeletal muscle, and the heart, where it is thought to contribute to neuronal plasticity\(^\text{24}\), muscle glycogen metabolism \(^\text{25}\), and cardiac contractility and excitability\(^\text{23,26}\). Moreover, I1 was implicated in the control of the activity of the Na-K-ATPase in the heart\(^\text{27}\), while PP1 was found to modulate the inhibitory effect of WNK4 on ROMK in the kidney\(^\text{28}\). Protein kinase A (PKA) phosphorylates I1 at a threonine residue in position 35 (T35), which activates I1 and makes it a strong and very specific inhibitor of PP1 with an IC\(_{50}\) value of 1nM\(^\text{29}\). Dephosphorylation of Thr35 by phosphatases such as PP2A and calcineurin (PP3) terminate the inhibitory action of I1\(^\text{26}\). Interestingly, I1 is critically involved in β-adrenergic and cAMP-dependent signaling in skeletal and heart muscle\(^\text{23,26}\) and I1 deficient mice are partially protected from isoprenaline induced cardiac remodeling and arrhythmia\(^\text{30}\).

Here, we tested the hypothesis that I1 is also critically involved in the cAMP/PKA-dependent stimulation of NCC phosphorylation. Using a variety of *ex vivo* approaches, we propose a novel signal transduction pathway in which cAMP-dependent
I1 mediates cAMP stimulation of NCC

phosphorylation and activation of I1 mediates the effect of cAMP-elevating hormones on NCC phosphorylation and hence activity.
I1 mediates cAMP stimulation of NCC

Materials and Methods

Reagents, cells and antibodies

Unless otherwise stated, reagents were purchased from Sigma Aldrich (Buchs, Switzerland). Calyculin A was purchased from Cell Signaling Technologies (Massachusetts, USA). 8-Br-cAMP, PKI 14-22 amide, myristoylated and H-89 were purchased from Tocris Bioscience (Bristol, UK). Endothall was purchased from EMD Millipore (Billerica, MA, USA).

\( tNCC, \ pT53NCC, \ pT58NCC \) and \( pS71NCC \) antibodies were previously described\(^21,22,31\). I1 antibody was purchased from Epitomics (California, USA. Cat. No: 1747-1). The phosphosite-specific antibody recognizing pT35I1 was obtained via affinity purification of serum from rabbits immunized with the phospho-peptide NH2-CRRRP(pT)PATL-CONH2 corresponding to mouse I1 (Pineda, Berlin, Germany). The specificity of the antibody was confirmed by immunohistochemistry (supplementary Fig 1). \( \beta \)-actin antibody was purchased from Sigma (Buchs, Cat. No: A5316 Switzerland). Rabbit anti FLAG Antibody was purchased from GenScript (New Jersey, USA, Cat No: A01868). Rabbit anti AQP1 antibody was previously described\(^32\). \( tNCC \) and \( pT58NCC \) antibodies used to detect calyculin A and endothall stimulation of NCC in MDCK type I cells were previously described (references \(^{33} \) and \(^{34} \) respectively). PP1c antibody was purchased from Abcam (Cambridge, UK, Cat No: 53315). Phospho-PKA substrate antibody was purchased from Cell Signaling Technologies (Massachusetts, USA, Cat. No: 9624). OSR1 antibody was purchased from Abcam (Cambridge, UK, Cat. No: 125468).

MDCK type I cells with tetracycline inducible FLAG-tagged NCC were previously characterized\(^{35}\).

Animals

All animal experiments were conducted according to Swiss Laws and approved by the veterinary administration of the Canton of Zurich, Switzerland (License numbers: 213/2015, and 185/2017). Experiments were conducted in male and female I1 deficient mice (I1-KO)\(^{24} \) or wildtype (WT). For automated DCT isolation, mice expressing the enhanced green fluorescent protein (EGFP) in the early segment of the DCT (DCT1) under the parvalbumin promoter (PV-EGFP) were used\(^{22} \). Both transgenic lines and WT animals were kept in a homogenetic C57Bl/6 background.
Mice were maintained in a 12/12 h light/dark cycle and had access to standard chow type 3430 purchased from Provimi-Kliba (Kaiseraugst, Switzerland) and water ad libitum. Animals were age, weight and sex matched for each experimental series.

**Kidney slices**

Sex, age, and weight matched mice were used for the preparation of kidney slices as described previously\(^\textsuperscript{18}\). To avoid confounding effects on NCC phosphorylation due to unequal dietary intake of K\(^+\), all mice were food deprived 16 hours prior to the experiment. 280 μm thick slices were incubated in Ringer-type solution for 30 minutes at 30.5°C for equilibration. The K\(^+\) concentration of the buffer was always 3 mmol/L. Stock solutions of isoproterenol, 3-isobutyl-1-methylxanthine (IBMX), calyculin A, PKI 14-22 amide, myristoylated, and forskolin were prepared in DMSO. Stock solutions of 8-Bromo-cAMP, Na\(^+\) salt, parathyroid hormone (PTH), norepinephrine (NE), and H-89 were prepared in H\(_2\)O. Equal volumes of either DMSO or H\(_2\)O were added as control vehicle when needed. After 30 minutes incubation with the drugs or vehicle solutions, slices were snap frozen in liquid nitrogen or immersion fixed with 3% PFA and processed for immunoblotting and histology, respectively. Electron microscopy confirmed that the structural integrity of DCT cells was preserved under the \textit{ex vivo} incubation of the tissue slices (supplementary Fig 2). Further experimental details can be found in the supplementary material and elsewhere\(^\textsuperscript{18}\).

**Immunoblotting**

Immunoblotting was performed as previously described\(^\textsuperscript{18}\).

**Statistics**

Unpaired Student’s t-test was used to compare two groups. For multiple comparison, one-way or two-way ANOVA followed by Tukey’s multiple comparison post-test was performed.

Experimental details of the following methods: yeast two hybrid, immunoprecipitation, immunofluorescence staining and fluorescence quantification, isolated perfused mouse kidney, electron microscopy and automated DCT isolation are included as supplementary material of this manuscript.
I1 mediates cAMP stimulation of NCC

**Results**

**cAMP-dependent stimulation of NCC phosphorylation is mediated by PKA**

First, we investigated whether an increase in intracellular cAMP levels stimulates NCC phosphorylation in native DCTs via a PKA-dependent pathway. We isolated EGFP-positive early DCT fragments (DCT1) from transgenic mice expressing EGFP under the control of the parvalbumin promoter (PV-EGFP)\(^22\). The isolated DCTs were incubated with a cocktail of the adenylate cyclase stimulator forskolin (FSK) (10 \(\mu\)mol/L) and the phosphodiesterase inhibitor IBMX (100 \(\mu\)mol/L) in the presence or absence of the PKA inhibitor PKI 14-22. Stimulation with FSK and IBMX triggered a significant increase in the phosphorylation of NCC accompanied with a mild increase in pSPAK-pOSR1 and a marked activation of PKA as monitored using a phospho-PKA substrate antibody (Fig 1). NCC phosphorylation and the anti-phospho-PKA substrate signal substantially diminished upon co-incubation with the PKA inhibitor PKI 14-22 (1 \(\mu\)mol/L). Under these conditions, the phosphorylation of SPAK and OSR1 remained almost unchanged compared to FSK/IBMX stimulation alone. Incubation of DCTs with 10 \(\mu\)M PKI 14-22 abolished the phosphorylation of NCC and of the SPAK-OSR1 kinases, indicating an overinhibition of the kinase pathway. Similar to PKI 14-22, the PKA inhibitor (H89) blocked the stimulatory effect of FSK/IBMX on NCC phosphorylation. Surprisingly and for unclear reasons, the effect of H89 was even more pronounced in cAMP-stimulated DCTs than in unstimulated DCTs (supplementary Fig 3).

**Genetic ablation of I1 attenuates the cAMP-dependent stimulation of NCC phosphorylation**

PKA phosphorylates I1 at position T35 *in vitro*, which renders I1 a potent and highly selective PP1 inhibitor\(^{29,36}\). To test whether I1 is critical for the cAMP-dependent stimulation of NCC, we analyzed kidney slices from WT and I1 deficient mice (I1-KO). Slices were incubated with either FSK, IBMX or the PKA-specific activator 8-Br-cAMP. All agonists strongly increased NCC phosphorylation at Thr 53 (Fig 2 A-C) and at Thr 58 and Ser 71 (Supplementary Fig 4) in kidney slices from WT mice. In contrast, FSK, IBMX, and 8-Br-cAMP had only a small effect on NCC phosphorylation in kidney slices from I1-KO mice (Fig. 2 A-C, and supplementary Fig 4).
I1 mediates cAMP stimulation of NCC

Protein phosphatase 1 interacts with and dephosphorylates NCC

Previous studies by us and others showed that all isoforms of the catalytic subunit of PP1 (Ppp1ca, Ppp1cb and Ppp1cc) are highly expressed in mouse\textsuperscript{22} and rat\textsuperscript{37} DCTs. Using a yeast-two-hybrid screen on a mouse total kidney library, we found that a NCC fragment comprising the first 133 amino acids of rat NCC interacts with PP1 (Ppp1cb, GI number 161484667) in addition to other known interacting partners (e.g. OSR1\textsuperscript{38}, SPAK\textsuperscript{39} and Hsp40\textsuperscript{40}). To further confirm that PP1 interacts with NCC, co-immunoprecipitation experiments were performed using lysates from MDCK type I cells stably transfected with a tetracycline-inducible FLAG-tagged NCC\textsuperscript{35}. As shown in figure 3A, endogenous PP1 was detected in samples immunoprecipitated with an anti-FLAG antibody but not in samples immunoprecipitated with an anti-AQP1 antibody or in the absence of antibody. Moreover, \textit{in vitro} experiments showed that the PP1 catalytic subunit $\alpha$ is able to dephosphorylate a synthetic peptide corresponding to the N-terminal tail of mouse NCC with a phosphorylated threonine at the position T58 (Fig 3B).

To confirm the functional relevance of PP1 for the regulation of NCC, NCC-expressing MDCK cells were also treated with PP1 and PP2A inhibitors. While the inhibition of PP1 with calyculin A profoundly stimulated NCC phosphorylation, the specific inhibition of PP2A with endothall did not change the phosphorylation of NCC (Fig 3C). Likewise, calyculin A increased NCC phosphorylation in kidney slices from both WT and I1-KO mice to the same extent (Fig 3D), indicating that the effect of calyculin A is downstream of the regulatory action of I1.

Norepinephrin and PTH stimulate NCC phosphorylation in a dose- and I1-dependent manner

It has been previously proposed that norepinephrine (NE) stimulates the phosphorylation of NCC via a PKA-dependent mechanism\textsuperscript{41}. Moreover, parathyroid hormone (PTH) was shown to activate the adenylate cyclase in the human and rat DCT promoting a strong increase in intracellular cAMP\textsuperscript{12,42}. Using kidney slices from WT and I1-KO mice, we tested whether these two hormones directly stimulate the phosphorylation of NCC in native DCTs and whether this effect depends on I1. As shown in figure 4 A and B, both hormones promote a dose-dependent increase in the phosphorylation of NCC in kidney slices from WT animals. The effect of NE on NCC
phosphorylation was completely abolished in I1-KO mice (Fig 4A). On the other hand, PTH still triggered a residual phosphorylation of NCC in kidney slices from I1-KO animals, although substantially weaker than in WT slices (Fig 4B).

**The β-adrenergic stimulation of NCC phosphorylation is mediated by I1**

Terker and coworkers suggested that β-adrenergic receptors are instrumental for the stimulation of NCC phosphorylation by catecholamines. We hypothesized that the β-adrenergic stimulation of NCC phosphorylation is mediated via I1. To test this hypothesis, kidney slices from WT and I1-KO mice were incubated with the β-adrenergic agonist isoproterenol. Isoproterenol caused a significant rise in NCC phosphorylation in kidney slices from WT mice (Fig 5A) in agreement with previous observations by us and others. However, this stimulatory effect was blunted in kidney slices from I1-KO mice, confirming the results with NE stimulation. Similar results were obtained using another ex-vivo model, namely the isolated perfused mouse kidney (Fig 5B).

Surprisingly, we did not observe any significant increase in the phosphorylation of SPAK-OSR1 upon stimulation with isoproterenol (Fig 5A). Moreover, when SPAK phosphorylation and activity was clamped at high levels by incubating the kidney slices in a low Cl⁻ solution (5 mmol/L), the stimulatory effect of isoproterenol on NCC was preserved and clearly additive to the effect of low Cl⁻ (supplementary figure 5). Moreover, neither the expression of SPAK (Fig 5A) nor of OSR1 (Supplementary figure 6) show any difference between WT and I1-KO kidneys. These findings suggest that at least in our experimental settings, the β-adrenergic stimulation of NCC is largely independent from an activation of the WNK/SPAK-OSR1 pathway.

**cAMP promotes I1 phosphorylation at threonine 35 (T35) in native DCTs**

To assess whether the activation of PKA promotes I1 phosphorylation in native DCTs, we developed a phosphoform-specific antibody against the PKA-phosphorylation site. This new pT35-I1 antibody recognizes specifically the phosphorylated form of I1 as demonstrated by peptide competition experiments in immunofluorescent studies (supplementary figure 1). Unfortunately, the antibody works only for immunofluorescent studies. Therefore, we assessed the phosphorylation levels of I1 by immunohistochemistry. Consecutive cryosections obtained from kidney slices incubated ex vivo either with vehicle or isoproterenol were stained with antibodies.
against total I1 (tI1), pT35I1, tNCC and pT53NCC (Fig 6) and the staining intensities in DCTs were then quantified using ImageJ software as described in the material and method section. As previously reported\textsuperscript{22}, I1 protein was found to be highly abundant in DCTs and in thick ascending limbs of Henle’s loop (TAL) (Fig 6A). In contrast to total I1, pT35I1 was barely detectable in DCTs in vehicle treated kidney slices. However, the signal for pT35I1 and also pT53NCC significantly increased in DCTs in kidney slices stimulated with isoproterenol (Fig 6A and B). Strikingly, the phosphorylation of I1 and NCC showed a strong linear correlation (Fig 6B). Of notice, both the total and the phosphorylated form of I1 were mainly seen at the apical cell surface of DCTs and hence in proximity to NCC (Fig 6A and C).
Discussion
The DCT is the target for several cAMP-elevating hormones including β-adrenergic agonists, PTH and vasopressin\textsuperscript{12}. These hormones are known to activate the DCT-specific NaCl cotransporter NCC but the involved signal transduction pathways remained poorly defined. In the present study, we used a set of \textit{ex vivo} approaches to reveal a complete signal transduction pathway by which cAMP-elevating hormones, via PKA, I1 and PP1 control NCC phosphorylation and hence activity.

In our studies, we tested the effect of two physiologically relevant hormones, namely norepinephrine and PTH. Both hormones strongly stimulated NCC phosphorylation in a dose-dependent manner in a range from 0.1 nmol/L to 100 nmol/L. For norepinephrine, this range matches well with the reference range of normal plasma norepinephrine concentrations in humans (i.e. 0.83-10 nmol/L)\textsuperscript{43}. For PTH, this range is above physiological levels (20-65 ng/L, \~{}2-6.5 pmol/L)\textsuperscript{44}. However, the PTH applied to native tissue elicited effects on NCC already at concentrations that were far below those reported in the literature (100 nmol/L) to activate NCC and TRPV5\textsuperscript{16,45} and to downregulate NaPi2a\textsuperscript{46} in cell systems and tissue slices, respectively. It is also important to consider that PTH is a peptide hormone that likely penetrates less efficiently into the tissue slices than the much smaller catecholamines. Moreover, the high levels of peptidases in the kidney slices (e.g. in the brush border of proximal tubules) may rapidly degrade PTH to inactive metabolites. Independent, from these possible technical hurdles, the data clearly indicate that both hormones are able to elicit a graded response of NCC phosphorylation. At least for norepinephrine, this graded response occurs in the range of physiological and pathophysiological plasma norepinephrine variations and may hence contribute to altered renal sodium reabsorption in response to changed sympathetic tone.

Several studies already suggested that cAMP-dependent activation of PKA contributes to the hormonal regulation of NCC\textsuperscript{13,15,41,47–49}. Nevertheless, as pointed out by Mutig et al. direct experimental support for the role of cAMP and PKA for the control of NCC activity in native DCTs had been limited probably due to the difficulty to establish readily available and suitable DCT cell models\textsuperscript{48}. Now, using isolated mouse DCTs and kidney slices incubated \textit{ex vivo} with forskolin and IBMX in the absence or presence of the PKA inhibitors PKI 14-22 and H-89, we provide compelling evidence that cAMP and PKA modify NCC activity in the native DCT. I1 is a direct target for PKA,
which phosphorylates I1 at a threonine in position 53 (pT35I1) and converts it into a potent and selective inhibitor of PP1. Consistent with an activation of the cAMP/PKA pathway in native DCTs, we observed a profound stimulation of I1 phosphorylation at the consensus PKA site (pT35) in response to isoproterenol stimulation. The I1 phosphorylation strongly correlated with the level of NCC phosphorylation suggesting that they are functionally linked. Interestingly, I1 appears to be barely phosphorylated at the PKA site (T35) in DCTs of vehicle-treated kidney slices suggesting that I1-dependent NCC regulation plays a significant role in response to hormonal stimuli but not under resting conditions.

In vitro kinase assays and experiments in heterologous expression systems suggested that PKA exerts its effects on NCC via the classical KHL3-WNK4-SPAK pathway. PKA-mediated phosphorylation of KLH3 at S433 decreases KLH3-dependent ubiquitination and degradation of WNK4. Likewise, PKA phosphorylates WNK4 at multiple sites including S64 and S1169, which finally promotes WNK4-dependent phosphorylation and activation of SPAK. PP1 was shown to modulate the phosphorylation levels of both WNK4 and SPAK. Studies on the regulation of the Na-K-2Cl cotransporter NKCC1, which is structurally related to NCC, suggested that PP1 binds to the N-terminal tail of NKCC1 in direct proximity to SPAK to dephosphorylate both SPAK and NKCC1. NCC lacks the amino acid motif mediating the binding of PP1 to NKCC1. Nevertheless, our yeast-two-hybrid and co-immunoprecipitation data suggest that PP1 and NCC do also interact and might be linked in a signaling complex that may involve also WNK4 and SPAK/OSR1. Therefore, it is conceivable that I1 and PP1 may control NCC activity directly via NCC-dephosphorylation and/or indirectly via controlling WNK4 and SPAK/OSR1 phosphorylation. However, neither in the current nor in our previous studies, we observed significant evidence for an involvement of I1 in SPAK/OSR1 regulation. The abundance and subcellular localization of total-SPAK, total-OSR1 (this study) and pSPAK/pOSR1 were similar in the kidneys and DCTs of wildtype and I1-KO mice. Moreover, FSK/IBMX and isoproterenol had no significant effects on SPAK/OSR1 phosphorylation in isolated DCTs (Fig 1) and in kidney slices (Fig 5), respectively. Likewise, incubation of the kidney slices in a low Cl−-solution, which clamps SPAK/OSR1 activity at high levels did not block the stimulatory effect of isoproterenol on NCC phosphorylation (suppl. Fig 5). Nevertheless, these negative results do not
formally exclude some activation of the WNK4/SPAK/OSR1 pathway. In fact, we consistently observed slight, but never statistically significant, increases in pSPAK/OSR1 immunoreactivities in tissue samples treated with FSK/IBMX and isoproterenol. Moreover, previous studies on heterologous expression systems linked SPAK to the cAMP/PKA-dependent NCC regulation\textsuperscript{13}, while studies on knockout mouse models implicated OSR1 (but not SPAK) in the catecholamine-dependent control of NCC\textsuperscript{15}. Part of these discrepancies may reflect the different experimental settings (e.g. in vitro, ex vivo, in vivo) and compounds used, but they may also indicate some redundancies in the signaling pathways, by which cAMP-elevating hormones stimulate NCC activity. In line with this view, we consistently observed some residual cAMP-dependent stimulation of NCC phosphorylation in kidneys of I1-KO mice in response to forskolin/IBMX and in particular in response to PTH. In contrast, the effect of catecholamines (isoproterenol and norepinephrine) on NCC phosphorylation appeared to depend almost exclusively on the presence of I1. A possible explanation for these differences might be a compartmentalization of the cAMP signaling pathways. In fact, cAMP reporter assays provided evidence for a spatial and temporal control of cAMP dynamics due to the presence of local micro-domains with proteins producing and degrading cAMP\textsuperscript{51}, which allow circumscribed effects independent from cAMP levels outside these microdomains\textsuperscript{52}. Thus, the hormone-induced cAMP/PKA-dependent activation of NCC may involve several redundant pathways including the previously characterized WNK4, SPAK and OSR1 kinase pathways\textsuperscript{15,35,48,53}. The current study adds I1 as an important additional regulator and suggests that in addition to the NCC-controlling kinases also the phosphatases are tightly regulated. Figure 7 shows the proposed signaling model that we believe best explains the current observations in the context of prior knowledge.

Aside from these novel insights into the molecular mechanism controlling NCC function, our findings may have some clinical implications. Inappropriately high sympathetic activity is thought to contribute to cardiovascular diseases including cardiac arrhythmia, cardiac failure, and arterial hypertension\textsuperscript{54}. Previous studies already implicated I1 in the β-adrenergic response of the heart modulating cardiac contractility\textsuperscript{55}, excitibility\textsuperscript{22}, and remodeling\textsuperscript{23}. The present study extends these observations to the kidney and shows that I1 is also critically involved in the renal response to catecholamines. Our data indicate that catecholamines increase the
phosphorylation of I1 at the PKA-consensus site T35, which convert I1 into a potent inhibitor of PP1-dependent NCC dephosphorylation. This finally increases NCC phosphorylation as observed in the present and several other\textsuperscript{15,41,56} but not all\textsuperscript{57} previous studies and may contribute to norepinephrine-induced salt sensitive hypertension \textsuperscript{58}. Interestingly, the pT35 site of I1 is dephosphorylated by PP3 (calcineurin) \textsuperscript{23}. Immunosuppressive therapy with calcineurin inhibitors such as tacrolimus and cyclosporine A is often complicated by the development of arterial hypertension, which was suggested to be linked to renal Na\textsuperscript{+} retention due to an activation of NCC \textsuperscript{19}. It is tempting to speculate that at least part of these effects are also mediated via I1. Future in vivo studies, will have to address the relevance of renal I1 for catecholamine- and calcineurin-induced arterial hypertension.

In summary, the present study identified the inhibitor 1 (I1) of protein phosphatase 1 as a central regulatory element in the signal transduction cascade that mediates the stimulatory effects of cAMP on the thiazide-sensitive NaCl cotransporter. I1 may represent an interesting point of convergence for different kinase and phosphatase pathways in the DCT contributing to the regulation of NCC in health and disease. Given its relevance for both the cardiac and renal response to β-adrenergic stimulation, I1 might also be an interesting drug target for the treatment of cardiovascular diseases, including arterial hypertension.
Author contributions:

DP, RAF, DLC and JL conceived and designed the study. DP, AW, SM, JC, LLR, FR, JRM, NF, RAF and DLC collected, analyzed and interpret the data. DP, RAF and JL wrote the manuscript. All authors approved the final version of the manuscript.

SM and AW contributed equally to this study.

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Disclosures

Authors have nothing to disclose
Supplemental material Table of content:

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Figure 1: PKA activation stimulates NCC phosphorylation in native DCTs. Left panel represents a typical immunoblot of isolated DCTs treated with vehicle, 10 μmol/L FSK + 100 μmol/L IBMX, FSK / IBMX + 1 μmol/L PKI 14-22 and FSK / IBMX + 10 μmol/L PKI 14-22. The activity of PKA was monitored using a phospho-PKA substrate antibody. Each lane corresponds to 400 DCT fragments. On the right, the densitometric analysis of pT53NCC/tNCC and pSPAK-pOSR1/tSPAK from 4 independent experiments ("n" in brackets) normalized to control vehicle group (red line) is represented. Error bars represent the standard error of the mean (SEM). *p<0.05 compared to control vehicle condition and assessed by one-way ANOVA followed by Tukey's multiple comparisons test.
**Figure 2:** PKA stimulation of NCC phosphorylation is impaired in I1-KO mice. 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 T53 in WT and I1-KO kidney slices. Bar charts represent the densitometric quantification of pT53NCC/tNCC normalized to control vehicle of each genotype from 6-9 slices ("n" in brackets) from 2-3 mice. Error bars represent the standard error of the mean (SEM). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 assessed by two-way ANOVA followed by Tukey’s multiple comparison post-test.
**Figure 3:** Protein Phosphatase 1 interacts with NCC and dephosphorylates it. **A:** FLAG IP pull down of NCC (left) and the catalytic subunit of PP1 (PPIc) (right). Unrelated anti-AQP1 antibody as well as no antibody were used as negative control. **B:** *in vitro* dephosphorylation of biotinylated pT58-mNCC peptide by PP1. **C:** Changes in NCC phosphorylation at T58 in MDCK type I cells with tetracycline inducible FLAG-tagged NCC expression upon treatment with calyculin A (left panel) or specific PP2A inhibitor endothall (right panel). Graph represents the densitometric quantification of immunoblots from 2 independent experiments *p*<0.05 by one-way ANOVA followed by Tukey’s multiple comparisons test. **D:** Changes in NCC phosphorylation upon treatment of WT and I1-KO mouse kidney slices with Calyculin A (20 nmol/L). Bar charts represent the densitometric quantification of pT53NCC/tNCC from 6 slices (in brackets) normalized to control vehicle of each genotype (2 mice per group). Error bars represent the standard error of the mean (SEM). ns: non-significant; **** *p*<0.0001 assessed by two-way ANOVA followed by Tukey’s multiple comparison post-test.
**Figure 4:** I1 mediates the effect of cAMP-increasing hormones on NCC phosphorylation. Dose-response effect of (A) Norepinephrine (NE) and (B) parathyroid hormone (PTH) on NCC phosphorylation (T53) in WT and I1-KO mouse kidney slices. Graphs represent the densitometric quantification of pT53NCC/tNCC normalized to control vehicle of each genotype (red line). The number of slices assayed from 1 (NE) or 2 (PTH) mice per genotype are in brackets. Error bars represent the standard error of the mean (SEM). Stars denote statistically significant differences (*p<0.05, **p<0.01, ****p<0.0001) between the two genotypes for the same hormone concentration assessed by unpaired Student’s t-test.
Figure 5: The β-adrenergic stimulation of NCC phosphorylation is impaired in 1-KO mice. A: Dose-response effect of β-adrenergic agonist isoproterenol on the phosphorylation of NCC (T53), SPAK and OSR1 in WT and I1-KO kidney slices. Graphs represent the densitometric quantification of the phosphorylation of pT53NCC/tNCC and pSPAK-pOSR1/tSPAK normalized to vehicle control of each genotype from 3-15 slices (in brackets) (1-5 mice), Stars represent statistical significance (*p<0.05, **p<0.01, ****p<0.0001) of the comparison between the two genotypes for the same concentration of isoproterenol assessed by unpaired Student’s t-test. B: β-adrenergic stimulation of NCC phosphorylation in isolated perfuse mouse kidneys. Bar charts represent the densitometric quantification of pT53NCC/tNCC normalized to control vehicle of each genotype in 4-5 mice / experiment (“n” in brackets). ns: non-significant; **p<0.01 assessed by two-way ANOVA followed by Tukey’s multiple comparison post-test. Error bars represent the standard error of the mean (SEM).
**Figure 6:** β-adrenergic stimulation promotes the phosphorylation of I1 at threonine 35 in native DCTs. **A:** Representative immunofluorescence stainings of tI1, pT35I1, tNCC and pT53NCC in consecutive sections of kidney slices from WT mice are shown. Slices were treated with isoproterenol 100 nmol/L or vehicle (scale bar 25 µm). **B:** The graph represents the relative mean intensity of pT35I1 staining (left panel) or pT53NCC (middle panel) in 6-7 slices (in brackets) from 3 mice (see supplementary methods for details). Error bars represent the standard error of the mean (SEM). *p<0.05, **p<0.01 assessed by unpaired Student’s t-test. Right panel represents the linear correlation between I1 (pT35) and NCC (pT53) phosphorylation. **C:** Higher magnification of typical pT35I1 staining in kidney slices treated with vehicle (left panel) and isoproterenol (right panel) highlighting its marked apical accumulation. (scale bar 25 µm)
Figure 7: Model of the stimulation of NCC phosphorylation by cAMP-increasing hormones. The cAMP-dependent activation of the WNK/SPAK/OSR1 pathway was shown in previous studies\textsuperscript{13,15,53}. The cAMP-dependent activation of the I1/PP1 pathway is presented in the current study.
### Panel A

| [NE] (nmol/L) | 0   | 0.1  | 1    | 10   | 100 |
|---------------|-----|------|------|------|-----|
| tNCC          |     |      |      |      |     |
| pT53NCC       |     |      |      |      |     |
| β-actin       |     |      |      |      |     |

#### WT

#### I1-KO

### Panel B

| [PTH] (nmol/L) | 0   | 0.1  | 1    | 10   | 100 |
|----------------|-----|------|------|------|-----|
| tNCC          |     |      |      |      |     |
| pT53NCC       |     |      |      |      |     |
| β-actin       |     |      |      |      |     |

#### WT

#### I1-KO
Mouse Kidney Slices

A

| [isoproterenol] (nmol/L) | 0  | 0.1 | 1  | 100 |
|-------------------------|----|-----|----|-----|
| tNCC                    |    |     |    |     |
| pT53NCC                 |    |     |    |     |
| tSPAK                   |    |     |    |     |
| pSPAK-pOSR1             |    |     |    |     |
| β-actin                 |    |     |    |     |

I1-KO

| [isoproterenol] (nmol/L) | 0  | 0.1 | 1  | 100 |
|-------------------------|----|-----|----|-----|
| tNCC                    |    |     |    |     |
| pT53NCC                 |    |     |    |     |
| tSPAK                   |    |     |    |     |
| pSPAK-pOSR1             |    |     |    |     |
| β-actin                 |    |     |    |     |

B

Isolated Perfused Mouse Kidney

|          | WT          | I1-KO      |
|----------|-------------|------------|
|          | vehicle     | iso        |
| tNCC     |              |            |
| pT53NCC  |              |            |
| β-actin  |              |      |
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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 (1 µg/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
I1 mediates cAMP stimulation of NCC 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. 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) 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 NaH2PO4, 2.5 CaCl2, 1.8 MgSO4, 10 glucose, pH 7.3). Renal cortex from both kidneys was dissected under a stereomicroscope. Samples were finely minced and digested in 20 ml of fresh
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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
elsewhere4.
Supplementary figure 1: Assessment of the specificity of the pT35I1 antibody by immunohistochemistry. A: Consecutive sections of kidneys from WT animals stained with pT35I1 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.
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**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.
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**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.
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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

|                | mean     | SEM      | n  | Two-way-ANOVA + Tukey’s multiple comparison                  | adjusted p value |
|----------------|----------|----------|----|-------------------------------------------------------------|-----------------|
| **FSK stimulation** |          |          |    |                                                             |                 |
| 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  |                                                              |                 |

|                | mean     | SEM      | n  | Two-way-ANOVA + Tukey’s multiple comparison                  | adjusted p value |
|----------------|----------|----------|----|-------------------------------------------------------------|-----------------|
| **IBMX stimulation** |          |          |    |                                                             |                 |
| 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 | 0.180904 | 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  |                                                              |                 |

|                | mean     | SEM      | n  | Two-way-ANOVA + Tukey’s multiple comparison                  | adjusted p value |
|----------------|----------|----------|----|-------------------------------------------------------------|-----------------|
| **8-Br-cAMP stimulation** |          |          |    |                                                             |                 |
| 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 | 1.114698 | 0.132396 | 3  |                                                              |                 |
| WT_vehicle_pS71NCC | 1.272917 | 0.166387 | 3  | WT vehicle vs 8-Br-cAMP                                    | 0.0008          |
| WT_8-Br-cAMP_pS71NCC | 2.193959 | 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  |                                                              |                 |
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**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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