

# Regulation of the epithelial Ca<sup>2+</sup> channel TRPV5 by reversible histidine phosphorylation mediated by NDPK-B and PHPT1

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**ABSTRACT** The kidney, together with bone and intestine, plays a crucial role in maintaining whole-body calcium (Ca<sup>2+</sup>) homeostasis, which is primarily mediated by altering the reabsorption of Ca<sup>2+</sup> filtered by the glomerulus. The transient receptor potential-vanilloid-5 (TRPV5) channel protein forms a six-transmembrane Ca<sup>2+</sup>-permeable channel that regulates urinary Ca<sup>2+</sup> excretion by mediating active Ca<sup>2+</sup> reabsorption in the distal convoluted tubule of the kidney. Here we show that the histidine kinase, nucleoside diphosphate kinase B (NDPK-B), activates TRPV5 channel activity and Ca<sup>2+</sup> flux, and this activation requires histidine 711 in the carboxy-terminal tail of TRPV5. In addition, the histidine phosphatase, protein histidine phosphatase 1, inhibits NDPK-B-activated TRPV5 in inside/out patch experiments. This is physiologically relevant to Ca<sup>2+</sup> reabsorption in vivo, as short hairpin RNA knockdown of NDPK-B leads to decreased TRPV5 channel activity, and urinary Ca<sup>2+</sup> excretion is increased in *NDPK-B*<sup>-/-</sup> mice fed a high-Ca<sup>2+</sup> diet. Thus these findings identify a novel mechanism by which TRPV5 and Ca<sup>2+</sup> reabsorption is regulated by the kidney and support the idea that histidine phosphorylation plays other, yet-uncovered roles in mammalian biology.

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## INTRODUCTION

The kidney plays an important role in the control of Ca<sup>2+</sup> homeostasis and secretion. Of the filtered Ca<sup>2+</sup>, only ~1–2% of the Ca<sup>2+</sup> filtered at the glomerulus is excreted in the urine (Hoenderop *et al.*, 2005). Although the majority of Ca<sup>2+</sup> is reabsorbed in the proximal tubule and thick ascending limb of the loop of Henle via paracellular pathways (Biner *et al.*, 2002), between 3 and 7% of the filtered Ca<sup>2+</sup> is reabsorbed in the distal convoluted tubule and collecting duct via the transient receptor potential-vanilloid-5 (TRPV5) channel (Boros *et al.*, 2009). TRPV5 in the apical plasma membrane provides a channel for Ca<sup>2+</sup> entry into the cell and its subsequent reabsorption

across the basolateral membrane via the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger NCX1 and Ca-ATPase PMCA1b (Boros *et al.*, 2009; Dimke *et al.*, 2011). Although TRPV5 is responsible for reabsorption of a relatively small portion of filtered Ca<sup>2+</sup>, it plays a critical role in maintaining whole-body Ca<sup>2+</sup> homeostasis. This is supported by the finding that TRPV5<sup>-/-</sup> mice exhibit urinary Ca<sup>2+</sup> wasting accompanied by secondary hyperparathyroidism (Hoenderop *et al.*, 2003).

TRPV5 is regulated by a number of mechanisms, which include transcriptional activation, intracellular trafficking, and posttranslational modification (Boros *et al.*, 2009; Dimke *et al.*, 2011). For example, parathyroid hormone (PTH), 1,25(OH)<sub>2</sub> vitamin D<sub>3</sub>, and estrogen increase TRPV5 expression via transcriptional activation of the TRPV5 gene (Hoenderop *et al.*, 2002; Boros *et al.*, 2009). PTH also increases TRPV5-mediated Ca<sup>2+</sup> influx by inhibiting endocytosis of TRPV5 from the plasma membrane (PM) by activating a protein kinase C pathway and activating TRPV5 channels in the PM by protein kinase A-mediated phosphorylation of threonine 709 in the carboxy-terminus (CT) of TRPV5 (Cha *et al.*, 2008; Boros *et al.*, 2009; de Groot *et al.*, 2009). Other receptors also regulate TRPV5 trafficking and TRPV5 channel activity via activation of downstream serine/threonine kinases. For example, the Ca<sup>2+</sup>-sensing receptor activates TRPV5 by stimulating phosphorylation of TRPV5 on serines 299 and

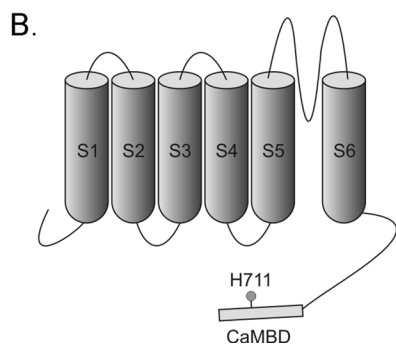
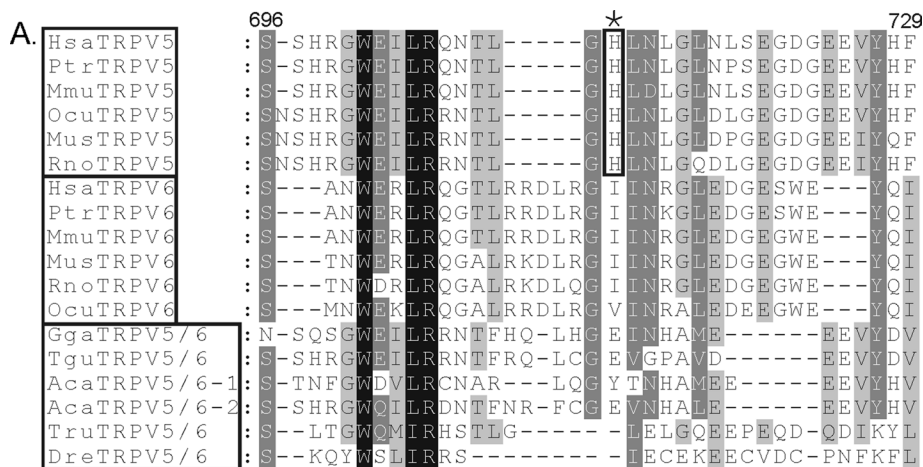
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Abbreviations used: KD, kinase dead; MDCK, Madin–Darby canine kidney; NDPK-B, nucleoside diphosphate kinase B; PHPT1, protein histidine phosphatase 1; shRNA, short hairpin RNA; TRPV5, transient receptor potential-vanilloid-5.

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**FIGURE 1:** (A) Alignment of amino acid sequence surrounding H711 of human TRPV5 with the corresponding sequences of TRPV5 and TRPV6 from a number of different species. (B) Schematic representation of TRPV5: the six transmembrane domains (S), the CT calmodulin-binding domain, and H711.

654 (Topala *et al.*, 2009), and tissue kallikrein, by activating the bradykinin receptor, increases TRPV5 activity by inhibiting endocytosis of TRPV5 from the PM in response to phosphorylation of the same serine residues (Gkika *et al.*, 2006).

We previously found that the K<sup>+</sup> channel KCa3.1 is activated by direct phosphorylation of histidine358 in the CT of KCa3.1 by nucleoside diphosphate kinase-B (NDPK-B) and is inhibited by the histidine phosphatase, protein histidine phosphatase 1 (PHPT1), which mediates dephosphorylation of the same histidine residue (Srivastava *et al.*, 2006, 2008). PHPT1 is an evolutionarily conserved, 14-kDa enzyme that dephosphorylates phosphohistidine, although it bears no resemblance to either serine/threonine or tyrosine phosphatases (Klumpp and Krieglstein, 2009). In searching the literature for other channels that resembled KCa3.1 and therefore might be candidates for being regulated in a similar manner, we identified TRPV5. Both channels are tetramers, bind calmodulin in the CT, and are regulated via binding of calcium to calmodulin (Maylie *et al.*, 2004; de Groot *et al.*, 2011). In addition, data demonstrate that mutation of histidine 711 (H711) in the CT of human TRPV5 leads to increase in TRPV5 channel activity that is mimicked by substitution of aspartic acid, an acidic amino for the same histidine (de Groot *et al.*, 2010), suggesting that phosphorylation of H711 could also activate TRPV5. An important role for H711 is also supported by the finding that H711 is evolutionarily conserved in TRPV5 and not in the related TRPV6 channel (Figure 1).

We show here that TRPV5 is regulated in a similar manner to KCa3.1. TRPV5 is activated by the histidine kinase NDPK-B and inhibited by the histidine phosphatase, PHPT1. Consistent with the

role for activation via direct histidine phosphorylation, NDPK-B activates TRPV5 inside/out patch experiments, and the increase in TRPV5 channel activity stimulated by NDPK-B is reversed by addition of PHPT1 or mutation of H711 on TRPV5 to alanine. Moreover, *NDPK-B*<sup>-/-</sup> mice exhibit increased renal Ca<sup>2+</sup> loss on a high-Ca<sup>2+</sup> diet. Thus, in addition to providing evidence for a new mechanism for regulation of TRPV5 and Ca<sup>2+</sup> homeostasis in vivo, these findings reinforce the importance of histidine phosphorylation in mammalian biology.

## RESULTS

### NDPK-B wild type, but not kinase-dead NDPK-B, activates TRPV5

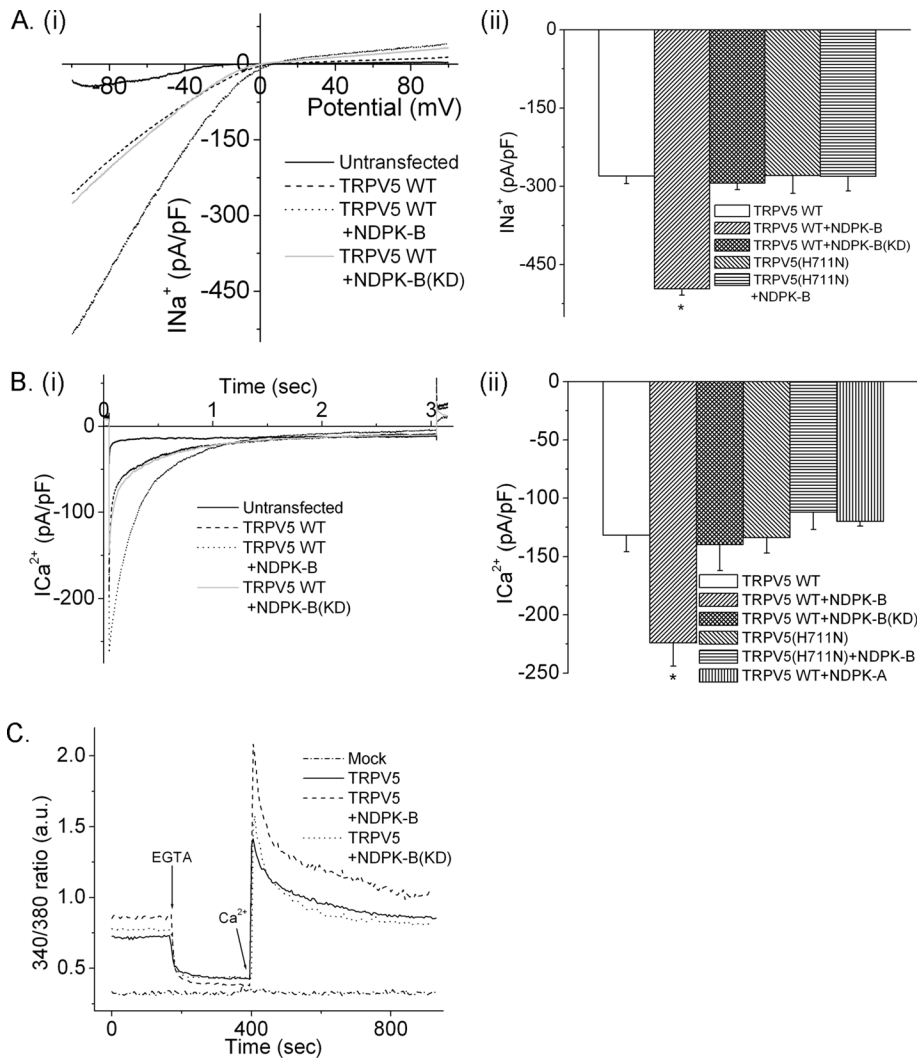
To assess whether NDPK-B activates TRPV5, we transfected HEK293 cells with TRPV5 either alone or together with NDPK-B(wild type [WT]) or a kinase-dead NDPK-B (NDPK-B(KD)) and assessed TRPV5 activity by whole-cell patch. HEK293 cells transfected with TRPV5 exhibited a characteristic inward rectifying Na<sup>+</sup> current that was absent in vector control-transfected cells (Figure 2A, i and ii). Cotransfection of NDPK-B(WT) with TRPV5 resulted in a marked increase in Na<sup>+</sup> current at -80 mV from 290 to 470 pA/pF (Figure 2A, i and ii). The increase in channel activity required NDPK-B kinase activity because the increase in Na<sup>+</sup> current was not seen in cells cotransfected with NDPK-B(KD) (Figure 2A, i and ii).

The increase in channel activity observed by cotransfection with NDPK-B(WT), but not NDPK-B(KD), suggested that NDPK-B activates TRPV5 by directly phosphorylating H711 in the CT of TRPV5. H711 was mutated to asparagine (TRPV5-H711N) and channel activity assessed in cells transfected with NDPK-B(WT) or NDPK-B(KD). Whereas basal activity of TRPV5(H711N) was similar to that of wild-type TRPV5 (TRPV5-WT) when transfected with a vector control, cotransfection with NDPK-B(WT) did not result in an increase in TRPV5(H711N) channel activity (Figure 2Aii).

NDPK-B(WT), but not NDPK-B(KD), also significantly increased TRPV5-mediated inward Ca<sup>2+</sup> current in HEK293 cells and decreased the feedback inhibition of TRPV5 mediated by Ca<sup>2+</sup> (Figure 2B, i and ii). Activation by NDPK-B(WT) is specific because we found that NDPK-A(WT), which is 88% identical to NDPK-B(WT), did not activate TRPV5 (Figure 2Bii). Consistent with the increase in Ca<sup>2+</sup> current, NDPK-B(WT), but not NDPK-B(KD), also led to an increase in TRPV5-mediated influx of Ca<sup>2+</sup> into whole cells as assessed with the Ca<sup>2+</sup>-sensitive dye fura-2-aceotymethyl ester (Fura-2-AM; Figure 2C). Thus, NDPK-B(WT) stimulates Ca<sup>2+</sup> influx into cells by TRPV5 by both increasing inward Ca<sup>2+</sup> current and decreasing the feedback inhibition mediated by Ca<sup>2+</sup>-induced association of calmodulin with CT of TRPV5 (de Groot *et al.*, 2011).

### NDPK-B activates TRPV5 channel activity at the plasma membrane

To assess whether NDPK-B activated TRPV5 by increasing the number of TRPV5 channels in the PM, we transfected HEK293 cells with green fluorescent protein (GFP)-TRPV5 alone or together with



**FIGURE 2:** NDPK-B activates TRPV5 channels. HEK293 cells were transfected with TRPV5(WT) or TRPV5(H711N) alone or together with NDPK-B(WT) or NDPK-B(KD), and (A)  $\text{Na}^+$  and (B)  $\text{Ca}^{2+}$  currents were determined by whole-cell patch clamp. (Ai) Representative *I/V* tracing in response to a voltage ramp. (Bi) Representative current-time graph. (Aii, Bii) Bar graph summaries of average  $\text{Na}^+$  (at  $-80$  mV) and  $\text{Ca}^{2+}$  current, respectively. Also shown in Bii is the inability of NDPK-A to activate TRPV5. Data are from  $n = 10$ – $15$  cells.  $*p < 0.05$  as compared with the current from TRPV5 WT-transfected cells analyzed using a one-way analysis of variance. (C) Cells transfected as in A were loaded with Fura-2-AM, and calcium influx was determined as described in *Materials and Methods*. Calcium flux experiments were done 10–15 times, and each time the data were obtained by averaging 30 cells.

mCherry-FLAG-NPDK-B and determined TRPV5 channels inserted in the PM after surface labeling of cells with biotin. These studies demonstrated that the amount of TRPV5 labeled with biotin was not increased in cells overexpressing NDPK-B (Figure 3A), indicating that NDPK-B does not increase TRPV5 channel activity by increasing the number of TRPV5 channels inserted into the PM.

We next tested whether NDPK-B directly increased the activity of TRPV5 channels in the PM in inside/out (I/O) patch experiments performed on patches obtained from HEK293 cells overexpressing TRPV5(WT) or TRPV5(H711N). The addition NDPK-B(WT) to I/O patches containing TRPV5(WT) led to a marked increase in TRPV5 channel activity, which was not observed after addition of NDPK-B(KD) (Figure 3, Ba and C). Consistent with the idea that NDPK-B directly activates TRPV5 by phosphorylating H711, the addition of the histidine phosphatase, PHPT1, reversed the increase in TRPV5

channel activity stimulated by NDPK-B (Figure 3, Ba and C). Moreover, NDPK-B(WT) failed to activate TRPV5(H711N) in similar I/O patch experiments (Figure 3, Bb and C).

### Endogenous NDPK-B is also required for full activation of TRPV5

To assess whether endogenous NDPK-B regulates TRPV5, we assessed TRPV5 channel activity after short hairpin RNA (shRNA) knockdown of NDPK-B in HEK293 cells. shRNA knockdown of NDPK-B led to decreased TRPV5-mediated  $\text{Na}^+$  (Figure 4A) and  $\text{Ca}^{2+}$  (Figure 4B) currents compared with cells transfected with an shRNA control. shRNA knockdown of NDPK-B also led to decreased TRPV5-mediated  $\text{Ca}^{2+}$  influx into cells (Figure 4C).

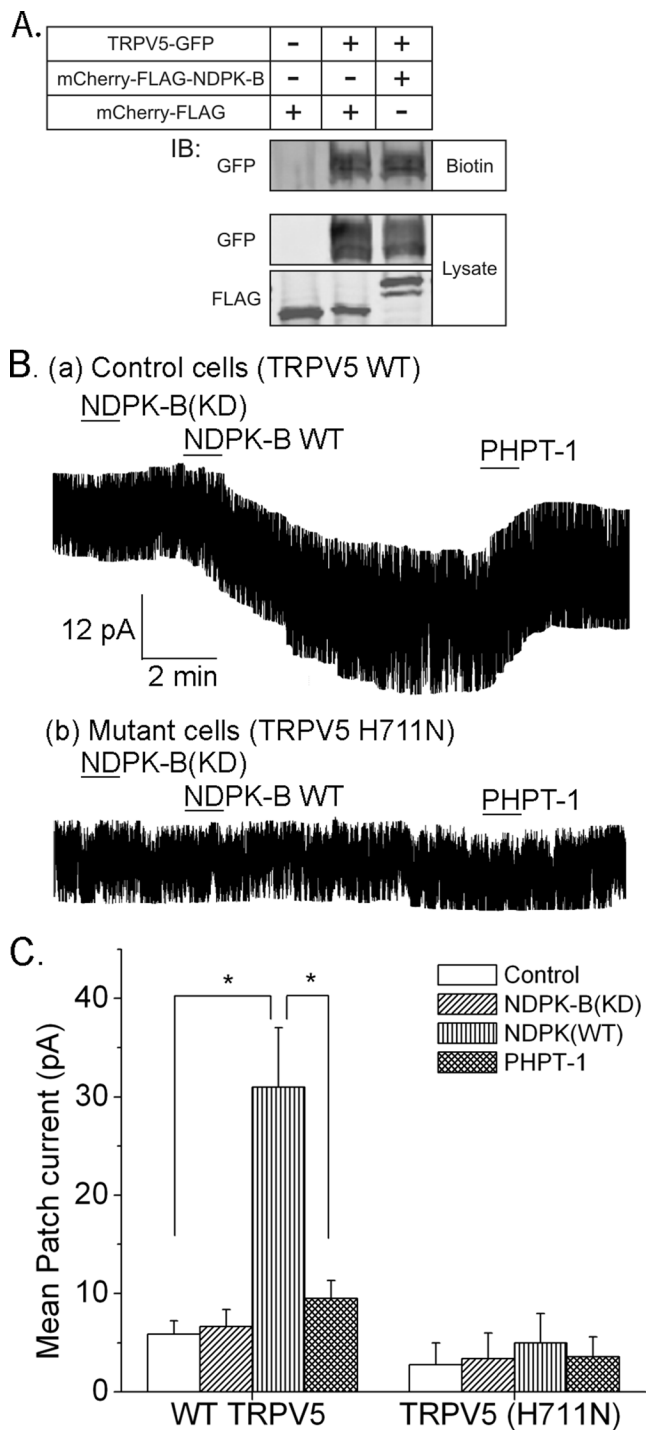
Madin–Darby canine kidney (MDCK) cells constitute a renal epithelial cell line derived from the distal/tubule collecting duct and are more physiologically relevant cells in which to study TRPV5 than HEK293 cells. Both  $\text{Na}^+$  current (Figure 5A) and TRPV5-mediated  $\text{Ca}^{2+}$  influx (Figure 5B) into MDCK cells decreased after knockdown of NDPK-B compared with cells transfected with shRNA control. Moreover, the decrease in  $\text{Na}^+$  current in shRNA-transfected cells could be rescued by overexpressing NDPK-B containing point mutations that abrogate binding of the shRNA, indicating that the shRNA inhibits by specifically silencing NDPK-B.

### NDPK-B-knockout mice have increased urinary $\text{Ca}^{2+}$ excretion on a high- $\text{Ca}^{2+}$ diet

NDPK-B-knockout mice are viable and appear phenotypically normal (Di *et al.*, 2010b). To assess whether NDPK-B contributes to  $\text{Ca}^{2+}$  reabsorption in vivo, we determined urinary  $\text{Ca}^{2+}$  excretion in *NDPK-B*<sup>-/-</sup> mice subjected to high- and low- $\text{Ca}^{2+}$  diets. Serum electrolytes, including  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{HCO}_3^-$ , blood urea nitrogen, creatinine, and phosphorus, were similar between *NDPK-B*<sup>+/+</sup> and *NDPK-B*<sup>-/-</sup> mice on both the low- and high- $\text{Ca}^{2+}$  diets. However, whereas excretion of urinary  $\text{Ca}^{2+}$  was similar between *NDPK-B*<sup>+/+</sup> and *NDPK-B*<sup>-/-</sup> mice on a low- $\text{Ca}^{2+}$  diet (Figure 6Ai),  $\text{Ca}^{2+}$  excretion on a high- $\text{Ca}^{2+}$  diet (Figure 6Aii), when normalized against urinary creatinine, was significantly increased in *NDPK-B*<sup>-/-</sup> mice. The increase in urinary  $\text{Ca}^{2+}$  was specific because under the same conditions the excretion of both  $\text{Na}^+$  and  $\text{K}^+$  was similar between *NDPK-B*<sup>+/+</sup> and *NDPK-B*<sup>-/-</sup> mice (Figure 6B and C).

## DISCUSSION

Histidine phosphorylation is the predominant mechanism for regulating signaling pathways in prokaryotes and some fungi and plants (Attwood *et al.*, 2007; Klumpp and Krieglstein, 2009; Kee and Muir, 2012). However, whereas for >40 years histidine phosphorylation has been proposed to play critical roles in regulating biological processes



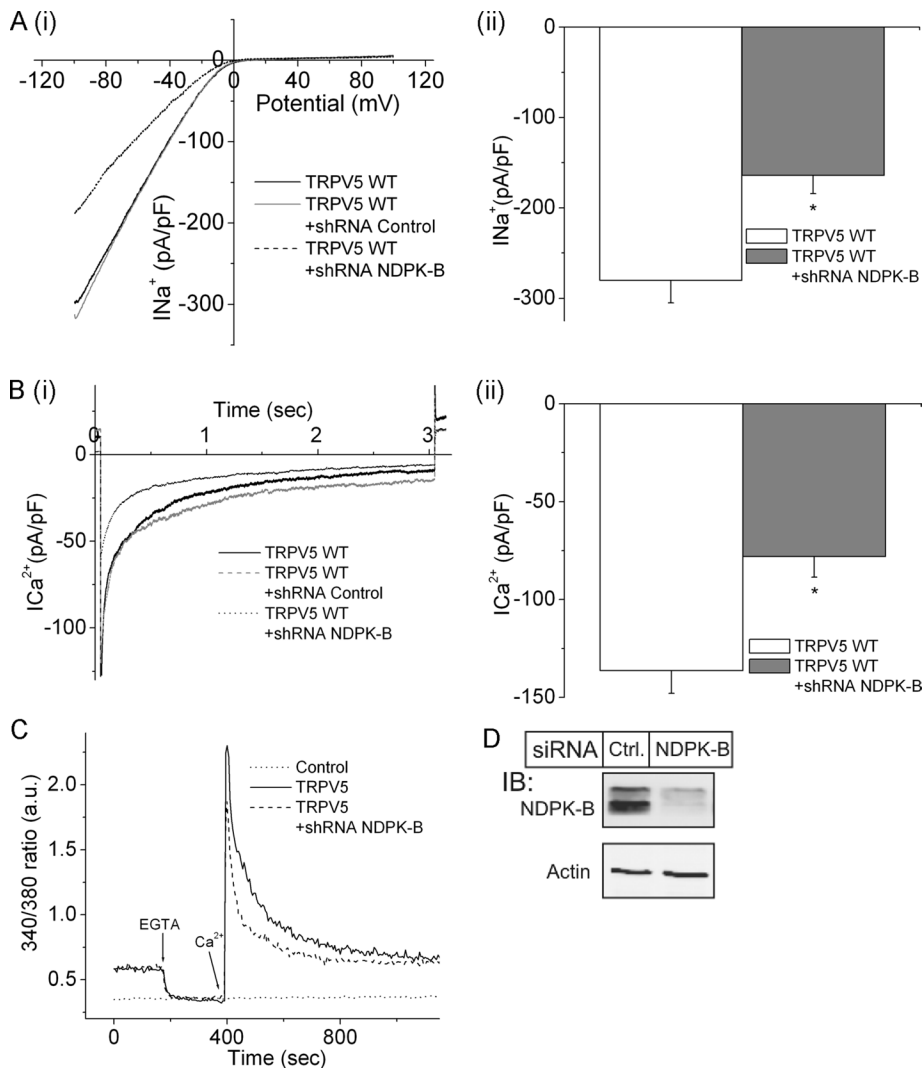
**FIGURE 3:** NDPK-B activates and PHPT1 inhibits TRPV5 in inside/out patch experiments. (A) HEK293 cells were transfected with GFP-TRPV5 together with mCherry-FLAG-NDPK-B or mCherry FLAG vector control. After surface labeling with biotin, cells were lysed, and biotin-labeled proteins were concentrated with High Capacity NeutrAvidin in Agarose Resin. Surface labeled GFP-TRPV5 was determined by immunoblotting precipitates with anti-GFP antibodies (top, biotin). To control for transfection efficiency, lysates were immunoblotted with anti-GFP or anti-FLAG antibodies as shown.  $n = 4$  experiments. (B) Inside/out patch experiments on patches derived from HEK293 cells transfected with TRPV5(WT) or TRPV5(H711N).  $\text{Na}^+$  current was measured before and after addition of proteins as indicated. (C) Summary of mean patch current from TRPV5(WT)- or TRPV5(H711N)-transfected cells.  $n = 10\text{--}12$  cells.  $*p < 0.05$ .

in mammals, until recently there has been little evidence that histidine phosphorylation also plays an essential role in mammalian biology (Attwood *et al.*, 2007, 2010; Klumpp and Krieglstein, 2009). Recent biochemical and genetic experiments demonstrating that NDPK-B phosphorylates and activates the  $\text{K}^+$  channel  $\text{KCa3.1}$  and the  $\beta$ -subunit of heterotrimeric G proteins has now provided definitive proof that histidine phosphorylation is also relevant to mammals (Cuello *et al.*, 2003; Srivastava *et al.*, 2006; Hippe *et al.*, 2009; Di *et al.*, 2010a,b). We now demonstrate that NDPK-B activates and PHPT1 reverses NDPK-B activation of TRPV5 channel activity, suggesting that TRPV5 is also regulated by reversible histidine phosphorylation.

NDPKs are encoded by the *NME* genes, which comprise 10 genes divided phylogenetically into two distinct groups, of which NDPK-A and -B (also known as Nm23-H1 and H2, respectively) are the best studied (Boissan *et al.*, 2009; Boissan and Lacombe, 2011). In addition, NDPK-A and -B have been shown to function as histidine kinases in mammalian cells and are the only histidine kinases molecularly identified in mammals (Klumpp and Krieglstein, 2009). For nucleoside diphosphate and histidine kinase activities, NDPK-A and -B autophosphorylate on histidine 118 to generate a high-energy phosphate intermediate, which is then transferred to a nucleoside diphosphate to generate a nucleoside triphosphate or to a histidine residue on another protein to generate phosphohistidine (Klumpp and Krieglstein, 2009). Whereas NDPK-A and -B are 88% homologous and share some redundant functions, NDPK-B has been shown to histidine phosphorylate specific targets and regulate specific biological processes that are not regulated by NDPK-A. For example, NDPK-B, but not NDPK-A, has been shown to histidine phosphorylate  $\text{KCa3.1}$  and the  $\beta$ -subunit of heterotrimeric G proteins (Hippe *et al.*, 2003; Srivastava *et al.*, 2006). We now show that NDPK-B, but not NDPK-A, also specifically activates TRPV5, and this activation requires both the NDPK-B histidine kinase activity and histidine 711 in the C-terminus of TRPV5.

We provide several lines of evidence that NDPK-B and PHPT1 directly regulate TRPV5 channels at the PM by reversible histidine phosphorylation and that this regulation is physiologically relevant. This is supported by the finding that NDPK-B wild type, but not a kinase-dead NDPK-B, activates TRPV5 in whole-cell patch experiments, and activation of TRPV5 by NDPK-B requires H711 in the CT of TRPV5. Surface labeling experiments demonstrate that NDPK-B does not affect the amount of TRPV5 at the PM. Instead, I/O patch experiments demonstrate that NDPK-B directly activates TRPV5 channels in the PM, and this activation is reversed after addition of the histidine phosphatase, PHPT1, or by mutating H711 on TRPV5 to alanine. Furthermore, the finding that shRNA knockdown of NDPK-B results in decreased TRPV5 channel activity in two cell lines, including MDCK cells, which are derived from renal distal convoluted tubule/collecting duct cells where TRPV5 functions, coupled with the increase in urinary calcium excretion in NDPK-B<sup>-/-</sup> mice, indicates that NDPK-B activation of TRPV5 is biologically relevant to calcium homeostasis *in vivo*. We have been unable to demonstrate direct phosphorylation of TRPV5 by NDPK-B in reconstitution experiments. We believe that this is likely due to technical problems with our assays, as well as reagents that are available to detect histidine phosphorylated proteins. With regard to NDPK-B, it was reported that NDPK-B requires an unidentified cofactor in order to act as a histidine kinase, which is likely why we have been unsuccessful in our reconstitution experiments (Cuello *et al.*, 2003).

Given the critical role TRPV5 plays in regulating whole-body calcium homeostasis (Hoenderop *et al.*, 2003), it is not surprising that TRPV5 is subject to tight regulation by a variety of factors. For example, PTH, CaSR, tissue kallikrein, and bradykinin have all been



**FIGURE 4:** shRNA knockdown of NDPK-B in HEK293 cells inhibits TRPV5 channel activity. NDPK-B was silenced by infecting HEK293 cells with an shRNA lentivirus to NDPK-B or a control shRNA, and (A)  $\text{Na}^+$  and (B)  $\text{Ca}^{2+}$  currents were measured as described in Figure 2. (C)  $\text{Ca}^{2+}$  flux measured in the same cells as described in Figure 1C. (D) Lysates from shRNA-infected cells were immunoblotted with antibodies to NDPK-B or  $\beta$ -actin, which served as loading control.  $n = 10\text{--}15$  cells.  $p < 0.05$  as compared with the WT.

shown to regulate TRPV5 by either affecting the trafficking of TRPV5 to and from the PM or directly regulating TRPV5 channel activity (Boros *et al.*, 2009; Dimke *et al.*, 2011). The signaling pathways that are activated downstream of these pathways that subsequently regulate TRPV5 activity are poorly defined, although PKC and PKA have been the predominant kinases shown to regulate TRPV5. Of interest, PTH-stimulated phosphorylation of threonine 709 in the CT of TRPV5 by PKA has been shown to increase the open probability of TRPV5 by diminishing the amount of  $\text{Ca}^{2+}$ -induced binding of calmodulin to TRPV5 (de Groot *et al.*, 2011); the rapid influx of  $\text{Ca}^{2+}$  leads to TRPV5 inactivation by promoting binding of calmodulin to TRPV5. Thus we speculate that NDPK-B phosphorylation of H711, which is contained within the amino acid sequence that mediates calmodulin binding to TRPV5, functions in a similar manner to increase TRPV5 channel activity.

We have only limited understanding of how NDPK-B is regulated in mammalian cells. Previous studies showed that generation of phosphatidylinositol 3-phosphate at the PM by the class II phos-

phatidylinositol 3-kinase (PI3-kinase) C2 $\beta$  is required for NDPK-B to phosphorylate and activate  $\text{KCa}_{3.1}$  in CD4 T-cells and mast cells (Srivastava *et al.*, 2009, 2012). Future studies will address whether PI3-kinase C2 $\beta$  is also required for NDPK-B activation of TRPV5 and whether any of the factors known to regulate TRPV5 channel activity mediate their effects by signaling via PI3-kinase C2 $\beta$  and/or NDPK-B. de Groot *et al.* (2010) reported that mutation of histidine 711 to aspartic acid (TRPV5(H711D)) led to increased TRPV5 channel activity. However, in contrast to our results, they found that the increase in TRPV5 channel activity of TRPV5(H711D) was predominantly due to an increase in total plasma membrane TRPV5 due to decreased endocytosis of the mutant channel. We do not have an explanation for these differences. One possibility is that NDPK-B activates TRPV5 via H711-dependent and -independent pathways. It is also possible that subtle differences in experimental conditions account for the different results. For example, under our experimental conditions, we found that both human and rabbit TRPV5(H711D) behave like wild-type TRPV5 when transfected into HEK293 or MDCK cells.

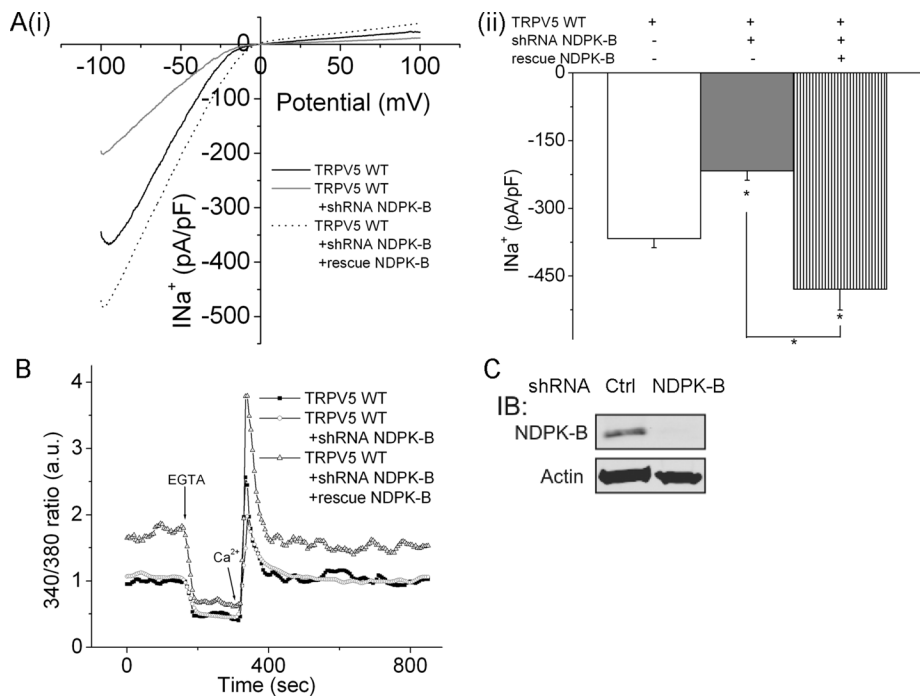
Protein phosphorylation is the most common and arguably best-studied mechanism for posttranslational modification. An overwhelming number of studies in mammalian systems have focused on serine/threonine and tyrosine phosphorylation, with relatively few studies addressing the role of histidine phosphorylation in mammalian biology. In addition to providing a new mechanism for the regulation of TRPV5 and  $\text{Ca}^{2+}$  homeostasis *in vivo*, our findings provide further justification to search for additional roles for histidine kinases and phosphatases in mammalian biology. It is anticipated that identification of signaling pathways regulated by

histidine phosphorylation will uncover new insights into mammalian biology and disease mechanisms and result in new therapeutic targets to treat disease.

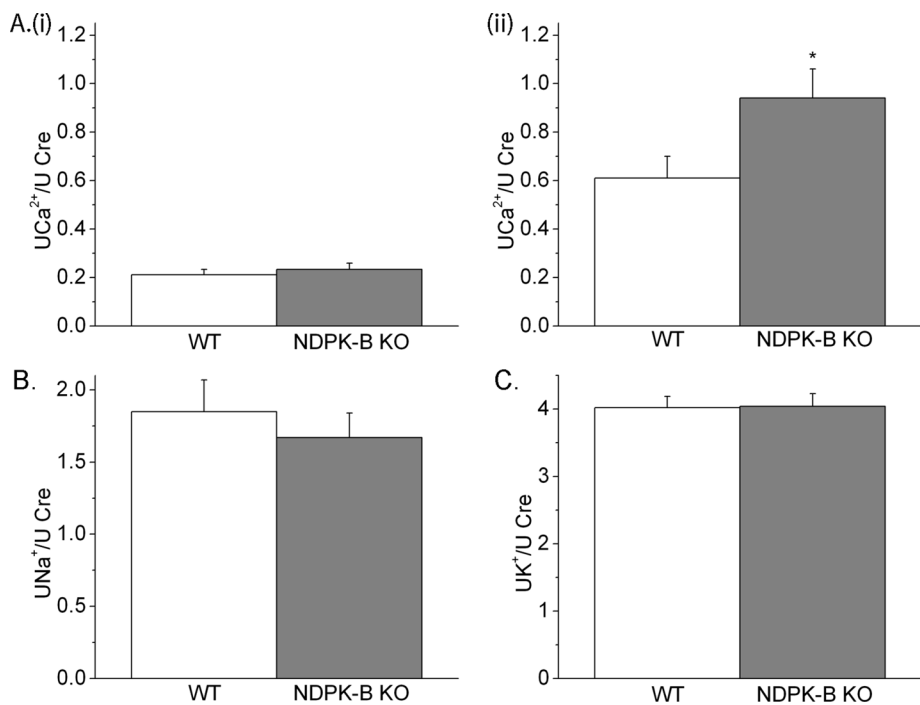
## MATERIALS AND METHODS

### DNA constructs, shRNA constructs, and cell culture

The human TRPV5 cDNA was kindly provided by Ji-Bin Peng (University of Alabama at Birmingham, Birmingham, AL; Zhang *et al.*, 2008) and was cloned into the pEGFP-N1 vector. Kinase-dead NDPK-B was generated by mutating histidine 118 on NDPK-B as previously described (Srivastava *et al.*, 2006). FLAG-NDPK-B was generated by cloning human NDPK-B into the mCherry-C1 vector. shRNA against NDPK-B were purchased from Sigma-Aldrich, St. Louis, MO (clone ID: TRCN0000054518), and lentiviruses were generated and used to infect cells. The shRNA-resistant NDPK-B clone contained the sequence CAGGTAGGACGAAACATC. The mutated nucleotides are underlined and did not alter the amino acid sequence. TRPV5H711N was generated using the following primer in



**FIGURE 5:** shRNA knockdown of NDPK-B in MDCK cells inhibits TRPV5 channel activity. NDPK-B was silenced in MDCK cells overexpressing GFP-TRPV5 as described in Figure 4, and (A) Na<sup>+</sup> current and (B) Ca<sup>2+</sup> flux were determined as described in Figures 2 and 4. (Aii) Bar graph summary of average Na<sup>+</sup> current at -80 mV. The decrease in Na<sup>+</sup> current after knockdown of NDPK-B was specific, as the Na<sup>+</sup> current in NDPK-B shRNA-infected cells could be rescued by expressing a mutant NDPK-B that did not bind the shRNA (A).  $n = 10$ –15 cells for each experiment.  $*p < 0.05$  as compared with the WT or as shown in the figure. (C) Lysates from shRNA- or control-transfected cells were immunoblotted with anti-NDPK-B or anti-actin antibodies.



**FIGURE 6:** NDPK-B<sup>-/-</sup> mice have increased urinary excretion of Ca<sup>2+</sup>. NDPK-B<sup>+/+</sup> or NDPK-B<sup>-/-</sup> mice were placed on low- or high-Ca<sup>2+</sup> diet for 2 wk, after which they were placed in metabolic cages and 24-h urine was collected. Shown is the amount of Ca<sup>2+</sup> in the urine on a low-Ca<sup>2+</sup> (Ai) or high-Ca<sup>2+</sup> (Aii) diet normalized to urinary creatinine. As a control, there was no difference in

the coding region (mutation underlined): ACCCTGGGGAACTTGAATCTT. cDNA constructs were transiently expressed in HEK293 cells by using Lipofectamine (Life Technologies, Grand Island, NY). Purified bacterial-expressed glutathione S-transferase (GST) fusions of full-length NDPK-B(WT), NDPK-B(KD), and histidine (His)-tagged PHPT1 have been described (Srivastava et al., 2006, 2008). NDPK-B (NME2) antibody is a mouse monoclonal (MO8) purchased from Abgent.

## Electrophysiology

**Whole-cell patch clamp.** Patch-clamp experiments were performed on single cells in the whole-cell configuration 24–48 h after transfection. Cells were plated on a 15-mm coverslip and positioned in a recording chamber (RC-25F; Warner Instruments), and patch clamping was performed on an IX51 Olympus microscope. An Axopatch 200B amplifier was used along with the Clampex10 software to record the currents. Membrane currents were filtered (-3 dB at 1 kHz) and digitized at 10 kHz (pClamp10 with Digidata1440A interface; Axon Instruments). AgCl was used a reference electrode. A voltage ramp of 450 ms was applied ranging from +100 to -100 mV every 5 s from a holding potential of +20 mV as previously described, with some modifications (de Groot et al., 2011). To measure Na<sup>+</sup> current, the internal pipette solution contained 140 mM KCl, 10 mM ethylene glycol tetraacetic acid, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2), and the external solution contained 140 mM NaCl, 5 mM EDTA, 1 mM MgCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES (pH 7.2). To measure Ca<sup>2+</sup> currents, 10 mM Ca<sup>2+</sup> (CaCl<sub>2</sub>) was added in the bath solution, and currents were measured for 3 s at -100 mV, stepping from a holding potential of +70 mV. Currents were not corrected for junction potential change.

**Inside/out patch.** TRPV5(WT) or TRPV5(H711N) was transfected into 293 cells. At 24–48 h posttransfection, TRPV5 channel activity was determined using I/O patch experiments as previously described (Cha et al., 2007). Briefly, the internal pipette solution contained 140 mM NaAsp, 10 mM

the amount of Na<sup>+</sup> (B) or K<sup>+</sup> (C) excreted in the urine when normalized to urine creatinine on the same samples from mice on a high-Ca<sup>2+</sup> diets.  $n = 10$  mice in each group. Statistics was done in Prism (GraphPad, La Jolla, CA), and t test was used for the significance.  $*p < 0.05$  as compared with the WT.

NaCl, 10 mM EDTA, and 10 mM HEPES, and membrane patches were excised in a bath solution containing 140 mM NaAsp, 10 mM NaCl, 1 mM EDTA, and 10 mM HEPES. Once a stable current was obtained, GST-NDPK-B(WT) or NDPK-B(KD) purified from bacteria (Srivastava *et al.*, 2006) was added to the I/O patch and channel activity recorded. To assess whether PHPT1 could reverse activation by of TRPV5, bacterial-expressed His-PHPT1 (Srivastava *et al.*, 2008) was added to I/O patch after addition of NDPK-B(WT).

### Cell surface biotinylation

HEK293 cells were transfected with GFP-TRPV5 with or without NDPK-B. At 24 h after transfection, cells were labeled with EZ Link Sulfo-NHS-SS-Biotin (Thermo Scientific, Rockford, IL) as previously described (van de Graaf *et al.*, 2008). After labeling, unreacted biotin was quenched with 100 mM glycine and 5% bovine serum albumin in phosphate-buffered saline, and cells were then lysed in lysis buffer containing 1% NP40. Biotinylated proteins were then precipitated using High Capacity NeutrAvidin Agarose Resin (Thermo Scientific Pierce). Cell surface TRPV5 was assessed by immunoblotting the avidin precipitates with anti-GFP antibodies.

### Calcium measurements

Cells were trypsinized and plated on 15-mm, round coverslips, and 24 h after transfection, cells were loaded with 5  $\mu$ M Fura-2-AM (Molecular Probes) in DMEM for 30 min at room temperature. Cells were washed and then resuspended in DMEM. Ca<sup>2+</sup> imaging was done with an IX81 epifluorescence microscope (Olympus) and OpenLab imaging software (Improvision). For single-cell analysis, 340/380 nm Fura-2-AM emission ratios of >50 cells/experiment were analyzed. Background fluorescence obtained from regions containing no cells was digitally subtracted from each image. Data are represented as the ratio 340/380 nm after background subtraction.

### NDPK-B<sup>-/-</sup> mice and urinary Ca<sup>2+</sup> determination

NDPK-B<sup>-/-</sup> mice that had been backcrossed 10 generations into C57Bl/6 mice have been described (Di *et al.*, 2010b). Littermate NDPK-B<sup>-/-</sup> or NDPK-B<sup>+/+</sup> mice were placed on standard (0.5%) or high-Ca<sup>2+</sup> (2%) diet from Lab Diet for 2 wk. The mice were then placed into metabolic cages, and 24-h urine samples were collected. Urine Ca<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup>, and creatinine concentrations were measured by Antech GLP (Morrisville, NC). All animal experiments were approved under Institutional Animal Care and Use Committee Protocol 110114.

### REFERENCES

Attwood PV, Ludwig K, Bergander K, Besant PG, Adina-Zada A, Kriegelstein J, Klumpp S (2010). Chemical phosphorylation of histidine-containing peptides based on the sequence of histone H4 and their dephosphorylation by protein histidine phosphatase. *Biochim Biophys Acta* 1804, 199–205.

Attwood PV, Piggott MJ, Zu XL, Besant PG (2007). Focus on phosphohistidine. *Amino Acids* 32, 145–156.

Biner HL, Arpin-Bott MP, Loffing J, Wang X, Knepper M, Hebert SC, Kaissling B (2002). Human cortical distal nephron: distribution of electrolyte and water transport pathways. *J Am Soc Nephrol* 13, 836–847.

Boissan M, Dabernat S, Peuchant E, Schlattner U, Lascu I, Lacombe ML (2009). The mammalian Nm23/NDPK family: from metastasis control to cilia movement. *Mol Cell Biochem* 329, 51–62.

Boissan M, Lacombe ML (2011). Learning about the functions of NME/NM23: lessons from knockout mice to silencing strategies. *Naunyn-Schmiedeberg Arch Pharmacol* 384, 421–431.

Boros S, Bindels RJ, Hoenderop JG (2009). Active Ca(2+) reabsorption in the connecting tubule. *Pflugers Arch* 458, 99–109.

Cha SK, Jabbar W, Xie J, Huang CL (2007). Regulation of TRPV5 single-channel activity by intracellular pH. *J Membr Biol* 220, 79–85.

Cha SK, Wu T, Huang CL (2008). Protein kinase C inhibits caveolae-mediated endocytosis of TRPV5. *Am J Physiol Renal Physiol* 294, F1212–F1221.

Cuello F, Schulze RA, Heemeyer F, Meyer HE, Lutz S, Jakobs KH, Niroomand F, Wieland T (2003). Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gbeta subunits. Complex formation of NDPK B with Gbeta gamma dimers and phosphorylation of His-266 IN Gbeta. *J Biol Chem* 278, 7220–7226.

de Groot T, Kovalevskaia NV, Verkaart S, Schilderink N, Felici M, van der Hagen EA, Bindels RJ, Vuister GW, Hoenderop JG (2011). Molecular mechanisms of calmodulin action on TRPV5 and modulation by parathyroid hormone. *Mol Cell Biol* 31, 2845–2853.

de Groot T, Lee K, Langeslag M, Xi Q, Jalink K, Bindels RJ, Hoenderop JG (2009). Parathyroid hormone activates TRPV5 via PKA-dependent phosphorylation. *J Am Soc Nephrol* 20, 1693–1704.

de Groot T, Verkaart S, Xi Q, Bindels RJ, Hoenderop JG (2010). The identification of histidine 712 as a critical residue for constitutive TRPV5 internalization. *J Biol Chem* 285, 28481–28487.

Di L, Srivastava S, Zhdanova O, Ding Y, Li Z, Wulff H, Lafaille M, Skolnik EY (2010a). Inhibition of the K+ channel KCa3.1 ameliorates T cell-mediated colitis. *Proc Natl Acad Sci USA* 107, 1541–1546.

Di L, Srivastava S, Zhdanova O, Sun Y, Li Z, Skolnik EY (2010b). Nucleoside diphosphate kinase B knock-out mice have impaired activation of the K+ channel KCa3.1, resulting in defective T cell activation. *J Biol Chem* 285, 38765–38771.

Dimke H, Hoenderop JG, Bindels RJ (2011). Molecular basis of epithelial Ca<sup>2+</sup> and Mg<sup>2+</sup> transport: insights from the TRP channel family. *J Physiol* 589, 1535–1542.

Gkika D, Topala CN, Chang Q, Picard N, Thebault S, Houillier P, Hoenderop JG, Bindels RJ (2006). Tissue kallikrein stimulates Ca(2+) reabsorption via PKC-dependent plasma membrane accumulation of TRPV5. *EMBO J* 25, 4707–4716.

Hippe HJ, Lutz S, Cuello F, Knorr K, Vogt A, Jakobs KH, Wieland T, Niroomand F (2003). Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gbeta subunits. Specific activation of Galpha by an NDPK B.Gbetagamma complex in H10 cells. *J Biol Chem* 278, 7227–7233.

Hippe HJ *et al.* (2009). The interaction of nucleoside diphosphate kinase B with Gbetagamma dimers controls heterotrimeric G protein function. *Proc Natl Acad Sci USA* 106, 16269–16274.

Hoenderop JG, Dardenne O, Van Abel M, Van Der Kemp AW, Van Os CH, St-Arnaud R, Bindels RJ (2002). Modulation of renal Ca<sup>2+</sup> transport protein genes by dietary Ca<sup>2+</sup> and 1,25-dihydroxyvitamin D3 in 25-hydroxyvitamin D3-1alpha-hydroxylase knockout mice. *FASEB J* 16, 1398–1406.

Hoenderop JG, Nilius B, Bindels RJ (2005). Calcium absorption across epithelia. *Physiol Rev* 85, 373–422.

Hoenderop JG *et al.* (2003). Renal Ca<sup>2+</sup> wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. *J Clin Invest* 112, 1906–1914.

Kee JM, Muir TW (2012). Chasing phosphohistidine, an elusive sibling in the phosphoamino acid family. *ACS Chem Biol* 7, 44–51.

Klumpp S, Kriegelstein J (2009). Reversible phosphorylation of histidine residues in proteins from vertebrates. *Sci Signal* 2, pe13.

Maylie J, Bond CT, Herson PS, Lee WS, Adelman JP (2004). Small conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels and calmodulin. *J Physiol* 554, 255–261.

Srivastava S, Cai X, Li Z, Sun Y, Skolnik EY (2012). Phosphatidylinositol-3-kinase C2beta and TRIM27 function to positively and negatively regulate IgE receptor activation of mast cells. *Mol Cell Biol* 32, 3132–3139.

Srivastava S *et al.* (2006). Histidine phosphorylation of the potassium channel KCa3.1 by nucleoside diphosphate kinase B is required for activation of KCa3.1 and CD4 T cells. *Mol Cell* 24, 665–675.

Srivastava S *et al.* (2009). The class II phosphatidylinositol 3 kinase C2beta is required for the activation of the K+ channel KCa3.1 and CD4 T-cells. *Mol Biol Cell* 20, 3783–3791.

Srivastava S, Zhdanova O, Di L, Li Z, Albaqumi M, Wulff H, Skolnik EY (2008). Protein histidine phosphatase 1 negatively regulates CD4 T cells by inhibiting the K+ channel KCa3.1. *Proc Natl Acad Sci USA* 105, 14442–14446.

Topala CN, Schoeber JP, Searchfield LE, Riccardi D, Hoenderop JG, Bindels RJ (2009). Activation of the Ca<sup>2+</sup>-sensing receptor stimulates the activity of the epithelial Ca<sup>2+</sup> channel TRPV5. *Cell Calcium* 45, 331–339.

van de Graaf SF, Rescher U, Hoenderop JG, Verkaart S, Bindels RJ, Gerke V (2008). TRPV5 is internalized via clathrin-dependent endocytosis to enter a Ca<sup>2+</sup>-controlled recycling pathway. *J Biol Chem* 283, 4077–4086.

Zhang W, Na T, Peng JB (2008). WNK3 positively regulates epithelial calcium channels TRPV5 and TRPV6 via a kinase-dependent pathway. *Am J Physiol Renal Physiol* 295, F1472–F1484.