

Original Article

# Regulation of CFTR chloride channel trafficking by Nedd4-2: role of SGK1

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## Abstract

**Introduction:** The cystic fibrosis transmembrane conductance regulator (CFTR) chloride ( $\text{Cl}^-$ ) channel is an essential component of epithelial  $\text{Cl}^-$  transport systems in many organs. CFTR is mainly expressed in the lung and other tissues, such as testis, duodenum, trachea and kidney. The ubiquitin ligase neural precursor cells expressed developmentally down-regulated protein 4-2 (Nedd4-2) has previously been shown to regulate abundance of several channel and carrier proteins in the plasma membrane, an effect reversed by glucocorticoid dependent kinase 1 (SGK1).

**Methods:** The present study was thus performed to elucidate the sensitivity of CFTR to regulation by Nedd4-2 and the serum and SGK1. To this end, the CFTR was heterologously expressed in oocytes alone or together with Nedd4-2 or the SGK1. The cRNAs encoding CFTR, Nedd4-2 and/or the constitutively active  $\text{S}^{422\text{D}}$ SGK1 have been injected into *Xenopus* oocytes. The activity of CFTR was measured by the two-electrode voltage-clamp technique and CFTR-mediated currents were elicited by the application of forskolin and IBMX (F/I).

**Results:** As a result, forskolin/IBMX treatment triggered cAMP-stimulated ion currents ( $I_{\text{cAMP}}$ ) in *Xenopus* oocytes expressing CFTR cRNA, but not in oocytes injected with water (control). Co-expression of Nedd4-2 markedly down-regulates the cAMP-stimulated ion current ( $I_{\text{cAMP}}$ ), an effect reversed by Co-expression of the constitutively active  $\text{S}^{422\text{D}}$ SGK1. In *Xenopus* oocytes co-expressing CFTR with  $\text{S}^{422\text{D}}$ SGK1 the cAMP-stimulated ion current ( $I_{\text{cAMP}}$ ) was similar to that in *Xenopus* oocytes expressing CFTR alone.

**Conclusion:** The present observations suggest that CFTR is a target for the ubiquitin ligase Nedd4-2, which is inactivated by the SGK1.

## Keywords:

CFTR;  
Trafficking;  
*Xenopus laevis* oocyte expression;  
SGK1;  
Nedd4-2

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## Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) forms a novel cAMP/protein kinase A (PKA) regulated chloride ( $\text{Cl}^-$ ) channel that is a basic element of epithelial  $\text{Cl}^-$  transport systems in several organs, including the intestines, pancreas,

lungs, sweat glands and kidneys (Sheppard and Welsh, 1999; Akabas, 2000; Lubamba et al., 2012). In the  $\text{Cl}^-$  secretory intestinal epithelium,  $\text{Cl}^-$  enters the epithelial cells via the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter (NKCC2) in the basolateral membrane and exits at the apical membrane through CFTR type anion channels (Kim and Steward, 2009). An important determinant of the rate of transepithelial  $\text{Cl}^-$  secretion

is the level of activation of CFTR trafficking and gating (Cuthbert et al., 1994), which depends on the extent and duration of its phosphorylation by protein kinases and phosphatases, which are regulated by a wide variety of hormones (Field and Semrad, 1993).

Since the cloning of the CFTR gene in 1989 (Riordan et al., 1989), the number of studies on its structure, function, regulation, biogenesis and degradation has grown exponentially (Higgins, 1992; Akabas, 2000; Corradi et al., 2015). CFTR mRNAs and proteins are abundantly expressed in the lung and other tissues, such as testis, duodenum, trachea and kidney (Crawford et al., 1991) and were shown to be expressed in the hypothalamus (Mulberg et al., 1998). The CFTR protein is usually localized on apical cell membranes but there is evidence that it is also expressed in the basolateral membrane of sweat duct epithelia (Bijman and Quinton, 1987; Reddy and Quinton, 1989).

Among the known regulators of membrane channel and carrier proteins trafficking are Nedd4-2, an ubiquitin ligase expressed in a wide variety of tissues (Anan et al. 1998) and SGK1, a serine/threonine kinase which is ubiquitously expressed (Lang and Stournaras, 2013). Nedd4-2 belongs to the family of homologous to E6-AP COOH-terminus (HECT) E3 ligases that catalyze the final step in the ubiquitination cascade (Chen et al., 2001). Nedd4-2 was shown to regulate and ubiquitinate the epithelial sodium channel ENaC (Debonneville et al. 2001, Snyder et al. 2002), the chloride channel ClC-5 (Hryciw et al., 2004), voltage gated sodium and potassium channels (van Bemmelen et al., 2004; Ekberg et al., 2007), the intestinal apical calcium entry channel TRPV6 (Zhang et al., 2010) and the cardiac potassium channel hERG1 (Albesa et al., 2011).

Serum and glucocorticoid-inducible serine/threonine protein kinase, SGK, was originally identified and cloned from rat mammary tumor cells (Webster et al., 1993). The kinase is expressed at high levels in all human tissues studied, including pancreas, liver, heart, lung, skeletal muscle, placenta, kidney and brain (Waldegger et al., 1997). Two novel isoforms of SGK, termed SGK2 and SGK3, have been previously described (Kobayashi et al., 1999). In mammalian cells SGK1 activity is regulated by phosphatidylinositol (PI) 3-kinase through phosphatidylinositol-dependent kinase (PDK) 1 and

2. SGK1 is not constitutively active but requires activation by phosphorylation. The upstream kinase is the phosphoinositol dependent kinase PDK1 which is in turn activated by IGF-1 through PI3 kinase (Kobayashi and Cohen, 1999; Park et al., 1999).

SGK regulates an increasingly large number of ion channels and transporters including ENaC (Wang et al., 2001; Faletti et al., 2002), voltage-gated (Kv) potassium (K<sup>+</sup>) channels (Gamper et al., 2002), NKCC2 (Fillon et al., 2001) and Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 3 (NHE3) (Yun et al., 2002). SGK indirectly regulates ENaC by phosphorylating and inhibiting the ubiquitin ligase Nedd4-2, which inhibits the degradation of internalized ENaC (Snyder et al. 2002).

The present study has been performed to determine the role of the Nedd4-2 and SGK1 in regulation of the CFTR trafficking and function. The result demonstrates Nedd4-2 down-regulates CFTR activity, and suggests a novel mechanism of CFTR regulation by SGK1 in *Xenopus laevis* oocyte expression system as it completely reverses the effect of Nedd4-2 on CFTR activity.

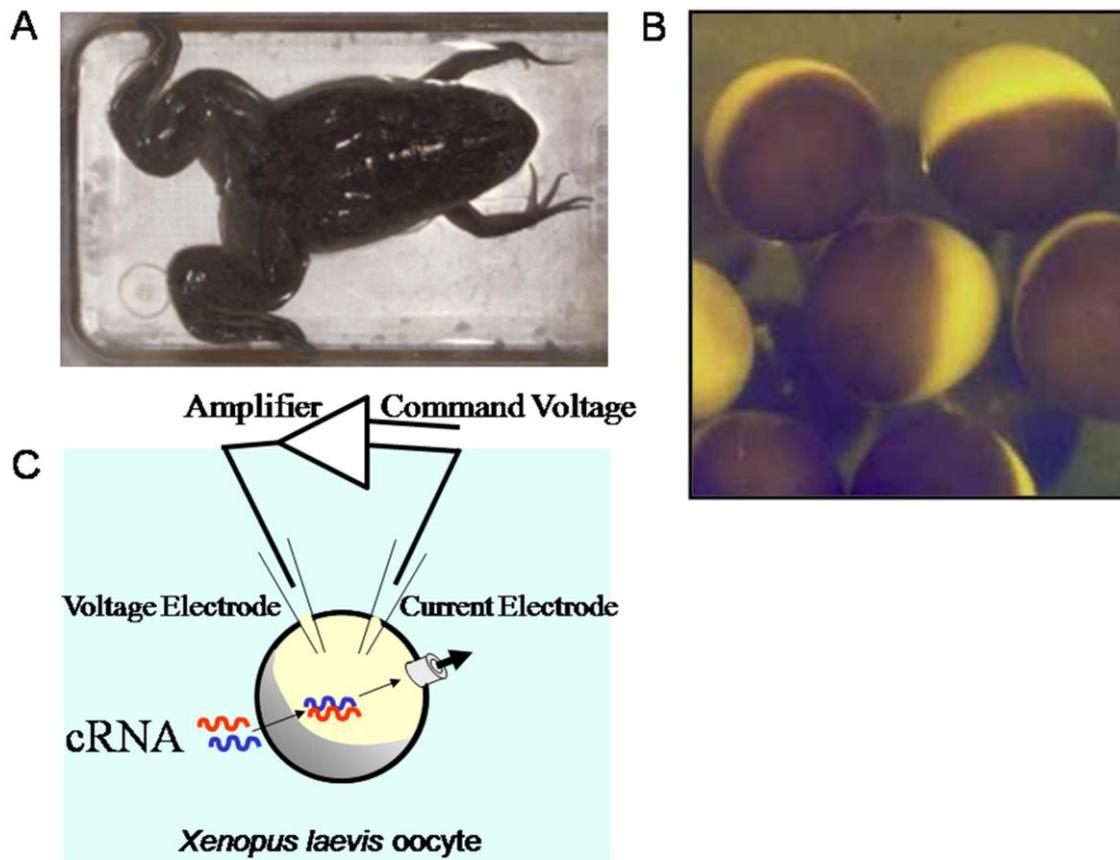
## Materials and methods

### cRNAs synthesis and characterization

cRNA encoding wild-type human CFTR (Riordan et al., 1989), wild-type human constitutively active S<sup>422D</sup>SGK1 (Kobayashi et al., 1999) and wild-type human Nedd4-2 (Debonneville et al. 2001) have been synthesized *in vitro* as described previously (Dieter et al., 2004; Gehring et al., 2009). Briefly, template cDNA was linearized with restriction enzymes KpnI (for CFTR), ClaI (for Nedd4-2) and Sall/StuI (for S<sup>422D</sup>SGK1). One microgram of linearized DNA was used to synthesize cRNA using *in vitro* transcription kits [mMessage mMachine T3 (for CFTR), T7 (for S<sup>422D</sup>SGK1) and Sp6 (for Nedd4-2); Ambion, Darmstadt, Germany]. The concentration of cRNA was evaluated using photospectrometry and transcript quality was checked by agarose gel electrophoresis.

### Expression of cRNAs in *Xenopus laevis* oocytes

Stage V-VI oocytes from *Xenopus laevis* (Fig. 1B) were prepared and cRNA injected as previously described in detail by Wagner et al. (2000). As shown in Fig. 1A, an adult female South African clawed frog



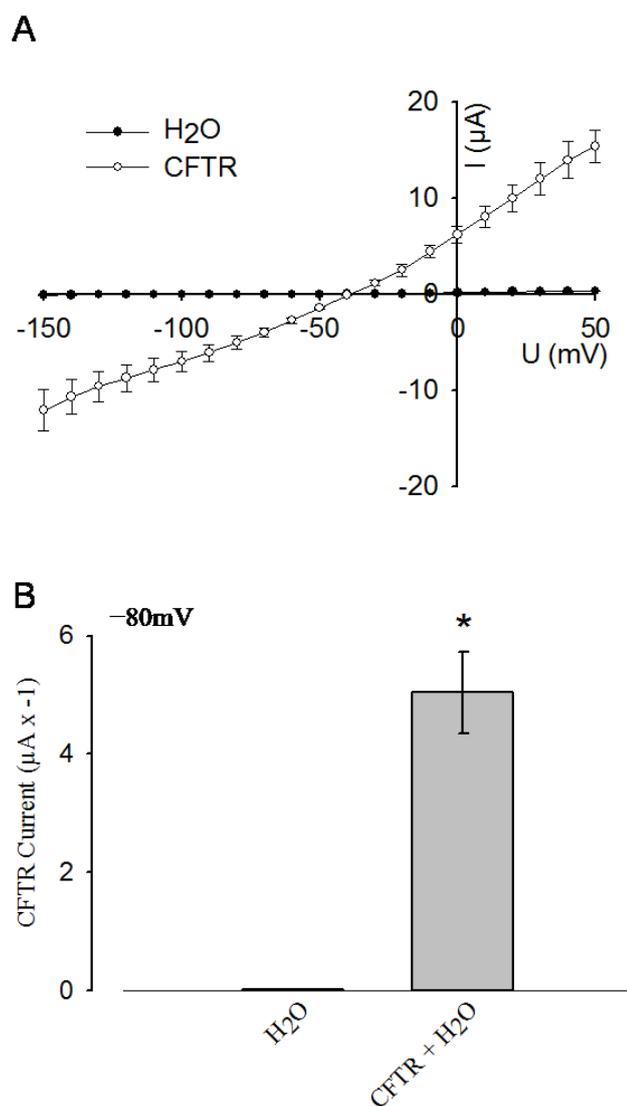
**Fig.1.** The voltage clamp recordings from *Xenopus laevis* oocytes. *A:* An adult female South African clawed frog (*Xenopus laevis*). *B:* stage V/VI *Xenopus laevis* oocytes. *C:* Two-Electrode Voltage-Clamp measurements are carried out by inserting two electrodes into the oocyte. The voltage electrode monitors the membrane potential; this is compared with a command voltage, and the magnified difference is applied to a current injection electrode. A bath electrode serves as the return path for the injected current.

(*Xenopus laevis*) was submersed in one liter of 3-aminobenzoic acid ethyl ester (0.1%) for about 15-30 min. After the frog was fully anesthetized it was placed on ice for surgery. A small abdominal incision (1 cm) was carried out and a segment of ovary was removed. Follicle membranes from isolated oocytes were enzymatically digested by treatment with an Oocytes-Ringer (OR-2) solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub> and 5 mM HEPES, pH 7.4) containing 1-2 mg/ml collagenase A for 2-2.5 h at room temperature with gentle agitation. The final solutions were titrated to the pH 7.4 using HCl or KOH. Only large oocytes (stage V or VI) were selected and stored overnight in a ND96 storage solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 5 mM HEPES, pH 7.4). The final solutions were titrated to the pH 7.4 using HCl or KOH. Each oocyte was injected 1 day after surgery with cRNA (50 nl total injection volume): 6 ng of CFTR, 5 ng of Nedd4-2 and 7.5 ng of SGK or

diethylpyrocarbonate (DEPC)-treated water (H<sub>2</sub>O). Oocytes injected with 50 nl DEPC-treated H<sub>2</sub>O served as controls. Injected oocytes were incubated at 18°C for 72 h in ND96 storage solution plus 2.5 mM sodium pyruvate and 50 µg gentamycin.

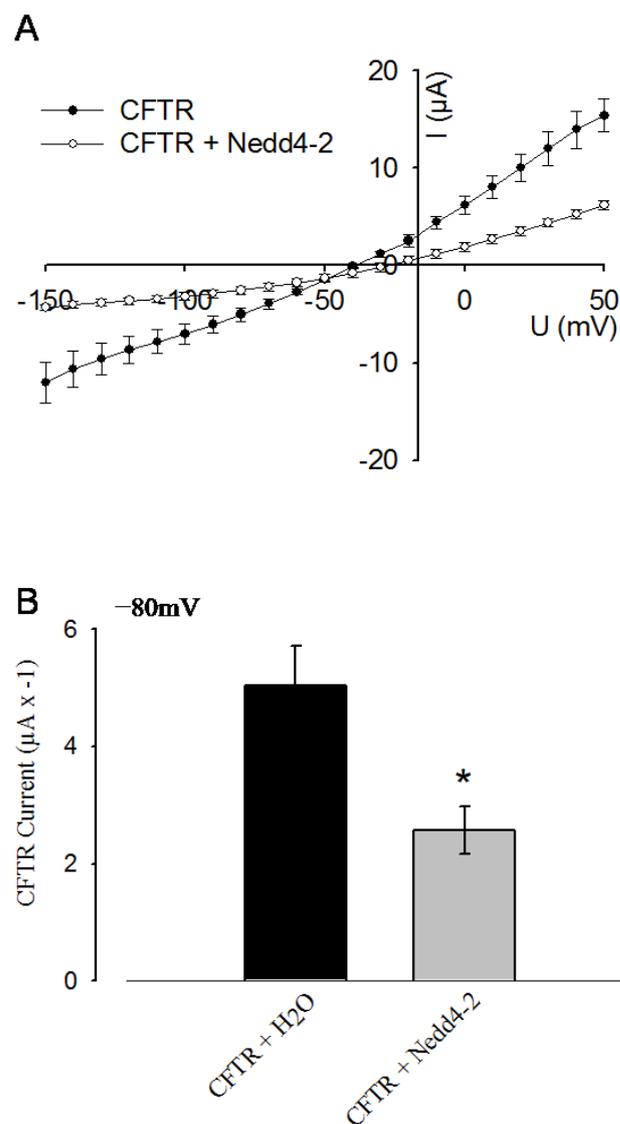
#### Voltage-clamp analysis

For recording, the oocytes were placed in a chamber at room temperature and continuously perfused with Ca<sup>2+</sup>-free ND96 solution (CaCl<sub>2</sub> was replaced with MgCl<sub>2</sub> and 0.5 mM EDTA was added), to minimize currents from Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels (Barish, 1983). As shown in Fig. 1C, CFTR-mediated Cl<sup>-</sup> currents were measured by the two-electrode voltage clamp technique three days post injection using a two-microelectrode voltage clamp system (NPI electronics, Tamm, Germany). Oocytes were impaled with glass electrodes containing 3 M KCl (resistances between 0.5-2 MΩ). The holding potential for each oocyte was the resting transmembrane potential (-30



**Fig.2.** The cAMP-activated CFTR current in *Xenopus* oocytes expressing human CFTR. *Xenopus laevis* oocytes were injected with either water (H<sub>2</sub>O) (50 nl, N=5) or human CFTR cRNA (6 ng, N=5). **A:** current-voltage (*I/V*) plots of cAMP-stimulated CFTR currents. **B:** summary of cAMP-stimulated CFTR currents at -80 mV. \* indicates statistically significant difference (\**P*<0.05).

mV). Voltages were increased stepwise in 10 mV increments from -150 mV to +50 mV adjusting for resting transmembrane potential. Currents were recorded at each voltage step both prior to and following 15 minutes of cAMP stimulation by 10 μM forskolin (an adenylyl cyclase activator) plus 1 mM 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor) in ND96 solution. In data analyses Cl<sup>-</sup> currents in the absence of IBMX were subtracted from IBMX-stimulated currents. Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (Munich, Germany).



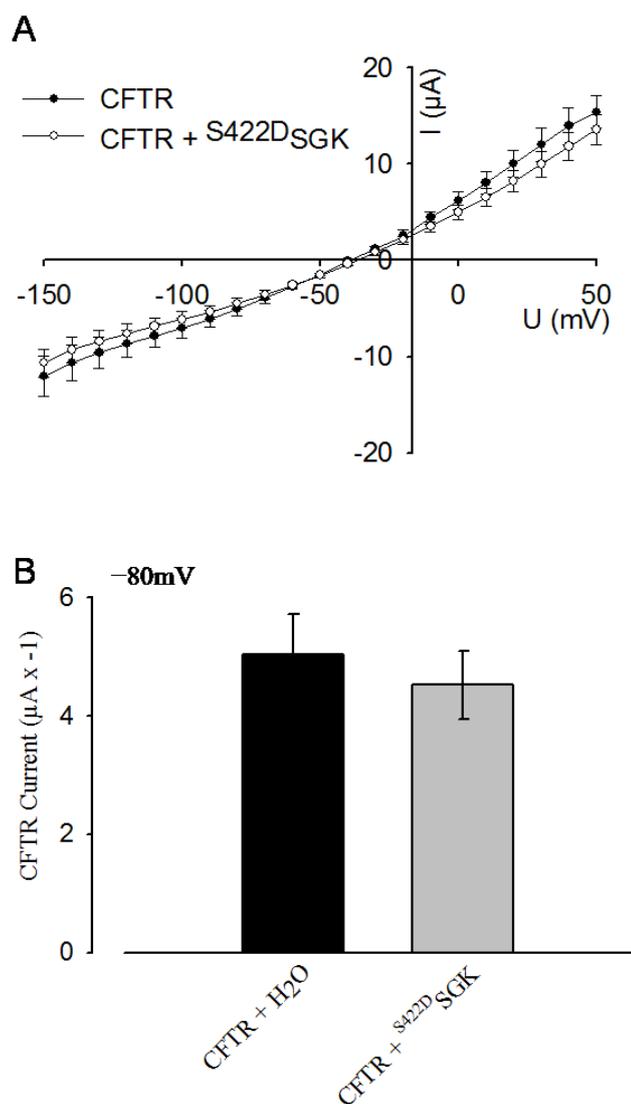
**Fig.3.** Reduction of CFTR channel activity by Nedd4-2. *Xenopus laevis* oocytes were injected with either CFTR cRNA (6 ng, N=5) or CFTR plus Nedd4-2 cRNAs (6 ng and 5 ng, respectively, N=4). **A:** current-voltage (*I/V*) plots of cAMP-stimulated CFTR currents. **B:** summary of cAMP-stimulated CFTR currents at -80 mV. \* indicates statistically significant difference (\**P*< 0.05).

### Statistical analysis

Data are provided as means ± SEM, *N* represents the number of oocytes investigated. The statistical significance of the various treatments on cAMP-stimulated CFTR currents was evaluated by comparing average currents at -80 mV with the Student's *t*-test and only results with *P*< 0.05 were considered as statistically significant.

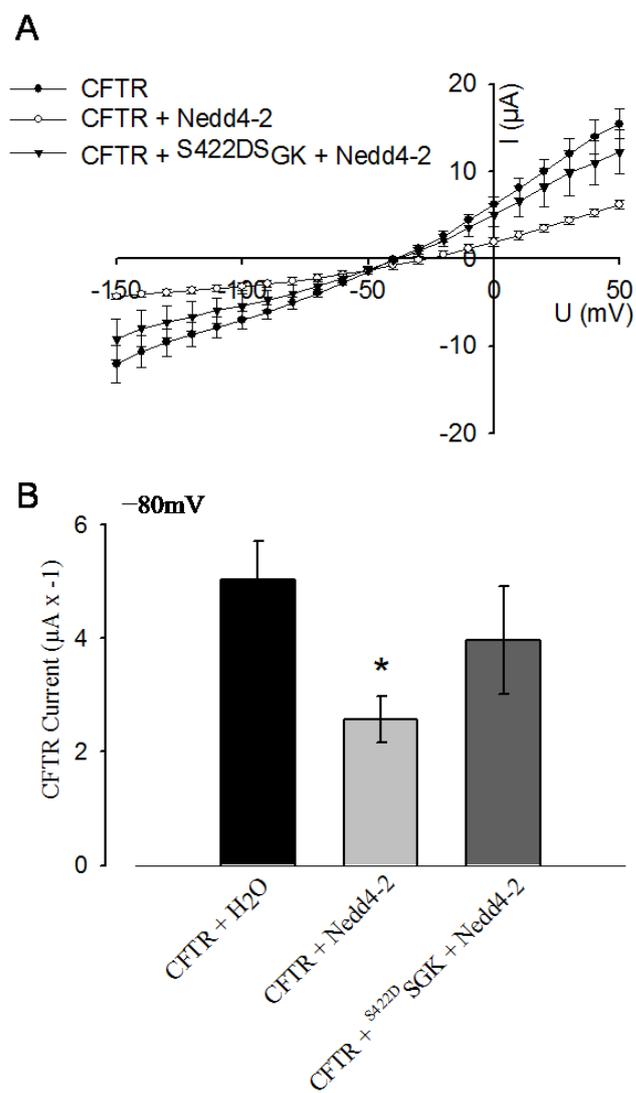
## Results

### CFTR-mediated Cl<sup>-</sup> currents in *Xenopus* oocytes



**Fig.4.** The effect of SGK1 on CFTR channel activity in *Xenopus oocytes* expressing system. *Xenopus laevis* oocytes were injected with either CFTR cRNA (6 ng,  $N=5$ ) or CFTR plus <sup>S422D</sup>SGK1 cRNAs (6 ng and 7.5 ng, respectively,  $N=5$ ). *A*: current-voltage ( $I/V$ ) plots of cAMP-stimulated CFTR currents. *B*: summary of cAMP-stimulated CFTR currents at  $-80$  mV.

Expression of human CFTR alone in *Xenopus laevis* oocytes increased cAMP-stimulated CFTR currents compared with  $H_2O$ -injected oocytes (Fig. 2). Inspection of the current voltage ( $I/V$ ) plots revealed that cAMP-stimulated CFTR currents had a linear  $I/V$  relationship with a reversal potential of  $-40$  mV, characteristics of CFTR-mediated  $Cl^-$  currents, as reported previously (Sato et al., 2007).  $H_2O$ -injected oocytes, not injected with human CFTR, did not show any electrogenic  $Cl^-$  transport and the cAMP-stimulated CFTR currents were negligible (Fig. 2A). The cAMP-stimulated CFTR current at  $-80$  mV was  $-5.037 \pm 0.684$   $\mu A$  in oocytes expressing human CFTR

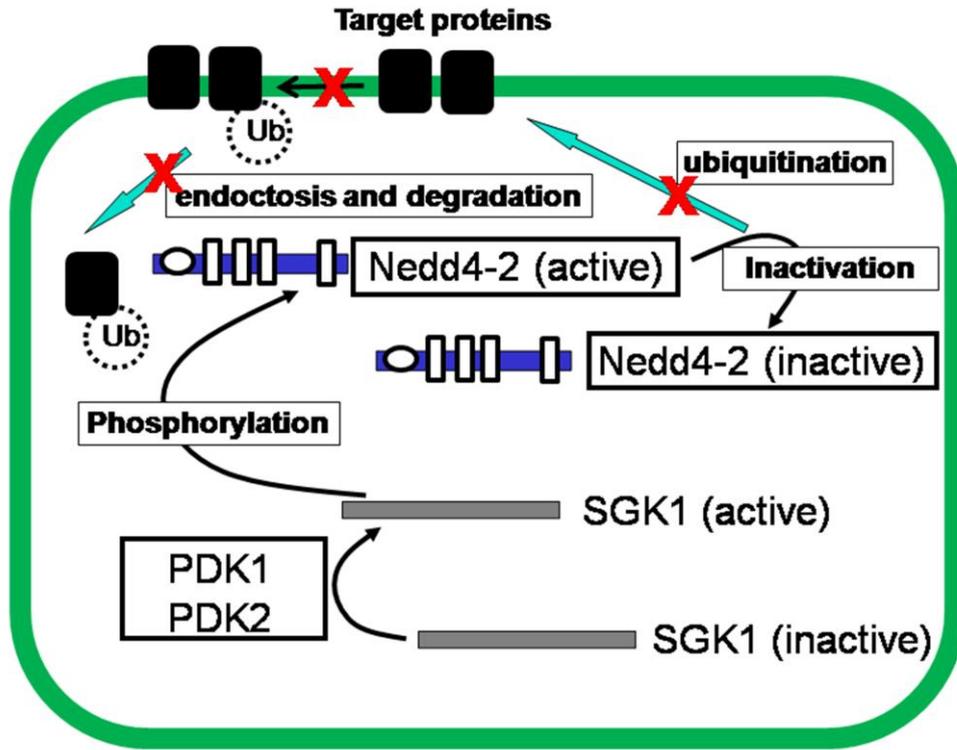


**Fig.5.** SGK1 reverses the effect of Nedd4-2 on CFTR channel activity. *Xenopus laevis* oocytes were injected with either CFTR cRNA (6 ng,  $N=5$ ), CFTR plus Nedd4-2 cRNAs (6 ng and 5 ng, respectively,  $N=4$ ) or CFTR plus <sup>S422D</sup>SGK1 and Nedd4-2 cRNAs (6 ng, 5 ng, and 7.5 ng, respectively,  $N=5$ ). *A*: current-voltage ( $I/V$ ) plots of cAMP-stimulated CFTR currents. *B*: summary of cAMP-stimulated CFTR currents at  $-80$  mV. \* indicates statistically significant difference ( $*P < 0.05$ ).

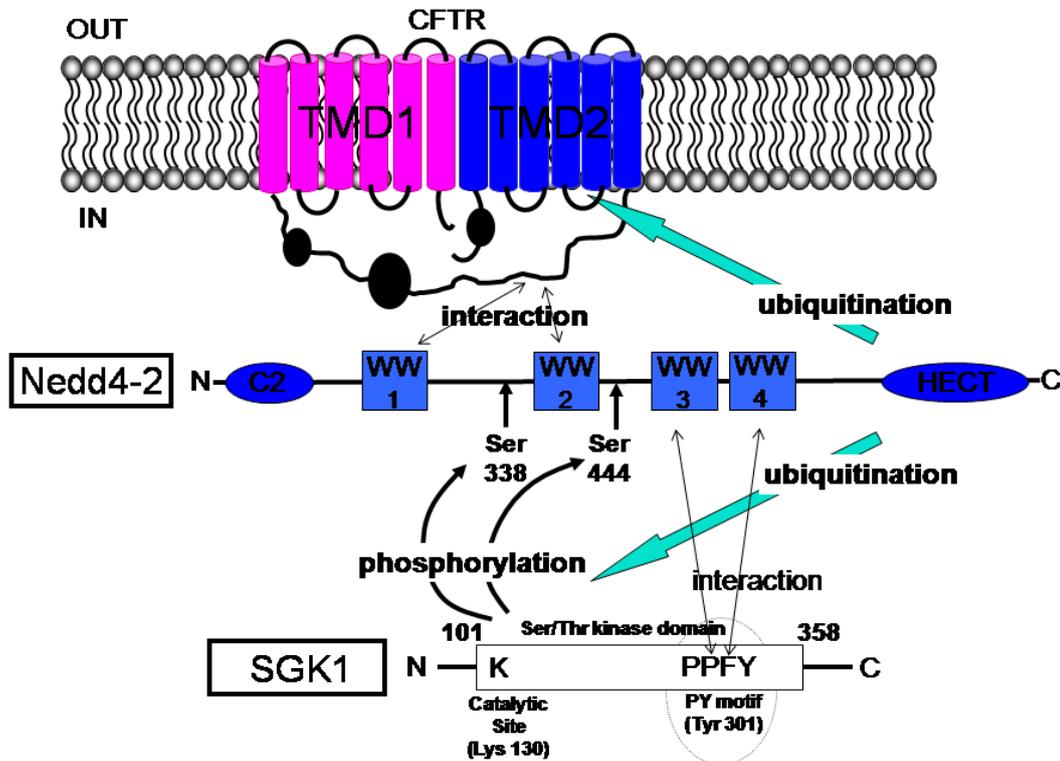
(Fig. 2B;  $N = 5$ ) and  $-0.012 \pm 0.005$   $\mu A$  in  $H_2O$ -injected oocytes (Fig. 2B;  $N = 5$ ).

#### Nedd4-2 decrease CFTR-mediated $Cl^-$ currents in *Xenopus oocytes*

As shown in Fig. 3, co-injection of Nedd4-2 with CFTR had effect on the cAMP-stimulated CFTR currents. The  $I/V$  plot for oocytes expressing CFTR and Nedd4-2 was decreased to the  $I/V$  plot of oocytes expressing CFTR alone (Fig. 3A). For example, the cAMP-stimulated CFTR current at  $-80$  mV was  $-5.037 \pm 0.684$   $\mu A$  in oocytes expressing CFTR (Fig.



**Fig.6.** A schematic view of Nedd4-2 and SGK1 phosphorylation and activation. The SGK1 activity is regulated by phosphatidylinositol (PI) 3-kinase through phosphatidylinositol-dependent kinase-1 (PDK1) and phosphatidylinositol-dependent kinase-2 (PDK2). The inhibitory effects of SGK1 on the ubiquitin ligase Nedd4-2 mediated by Nedd4-2 phosphorylation and inactivation.



**Fig.7.** A schematic model of interaction between CFTR, SGK1 and Nedd4-2. The SGK1 phosphorylated Nedd4-2 and thus inactivated the ubiquitin ligase.

3B;  $N = 5$ ) and  $-2.567 \pm 0.404 \mu\text{A}$  in oocytes expressing CFTR and Nedd4-2 (Fig. 3B;  $N = 4$ ).

### **SGK1 does not stimulate CFTR-mediated $\text{Cl}^-$ currents in *Xenopus* oocytes.**

*Xenopus* oocytes were used as a model to assess whether SGK1 alone up-regulates CFTR-mediated  $\text{Cl}^-$  currents. If SGK1 stimulates CFTR and thereby enhances its phosphorylation; then co-expression of SGK1 should increase cAMP-stimulated CFTR currents. Co-injection of the constitutively active form of SGK1 ( $\text{S}^{422\text{D}}$ SGK1) with CFTR had no effect on the cAMP-stimulated CFTR currents (Fig. 4). The  $I/V$  plot for oocytes expressing CFTR and  $\text{S}^{422\text{D}}$ SGK1 was not significantly different to the  $I/V$  plot of oocytes expressing CFTR alone (Fig. 4A). cAMP-stimulated CFTR current at  $-80 \text{ mV}$  was  $-5.037 \pm 0.684 \mu\text{A}$  in oocytes expressing CFTR alone (Fig. 4B;  $N = 5$ ) and  $-4.516 \pm 0.573 \mu\text{A}$  in oocytes expressing CFTR plus  $\text{S}^{422\text{D}}$ SGK1 (Fig. 4B;  $N = 5$ ).

### **SGK1 reverses the effect of Nedd4-2 on CFTR-mediated $\text{Cl}^-$ currents in *Xenopus* oocytes**

As shown in Fig. 5, upon co-expression of CFTR with both, SGK1 and Nedd4-2, cAMP-stimulated CFTR currents were not significantly different in *Xenopus* oocytes co-expressing CFTR alone or together with both,  $\text{S}^{422\text{D}}$ SGK1 and Nedd4-2 compared with oocytes expressing CFTR with Nedd4-2 (Fig. 5A). The cAMP-stimulated CFTR current at  $-80 \text{ mV}$  was  $-5.037 \pm 0.684 \mu\text{A}$  in oocytes expressing CFTR (Fig. 5B;  $N = 5$ ),  $-2.567 \pm 0.404 \mu\text{A}$  in oocytes expressing CFTR and Nedd4-2 (Fig. 5B;  $N = 4$ ) and  $-3.970 \pm 0.951 \mu\text{A}$  in oocytes expressing CFTR together with both  $\text{S}^{422\text{D}}$ SGK1 and Nedd4-2 (Fig. 5B;  $N = 5$ ).

## **Discussion**

CFTR-mediated anion and fluid secretion is vital for the survival and function of many fluid and  $\text{Cl}^-$  transporting epithelia and mutations in CFTR gene cause CF, a severe, potentially and even life-threatening genetic disease (Riordan et al., 1989; Cheng et al., 1990; Levy and Farrell, 2015). On the other hand, some enteric bacteria produce toxins that may increase the number of CFTR channels that get inserted into the apical membrane of intestinal epithelium, resulting in secretory diarrhea (Li et al., 2005; Sonawane et al., 2006; Lee et al., 2007).

Therefore, activation of CFTR trafficking and gating is a physiologically important mechanism for maintaining normal epithelial cell function.

Recent experiment suggests that expressed wild-type SGK1 is not constitutively active but require activation (Andres-Mateos et al., 2013). The kinases are activated through a signaling pathway involving PI3-kinase and phosphoinositide-dependent kinase (Kobayashi and Cohen, 1999). Obviously, the activation of the kinases is not limiting in oocytes. In earlier studies, wild-type SGK1 has been shown to be similarly effective as constitutively active  $\text{S}^{422\text{D}}$ SGK1 (Lang et al., 2000).

In earlier studies, Sato et al. (2007) reported that wild-type SGK1, but not constitutively active form of SGK1 ( $\text{S}^{422\text{D}}$ SGK1), stimulated CFTR mediated  $\text{Cl}^-$  currents in *Xenopus* oocytes. However, further studies are needed to clarify the mechanism whereby SGK increased CFTR  $\text{Cl}^-$  currents in *Xenopus* oocytes. Because SGK increases the plasma membrane expression of other ion channels, the goal of this paper was to test the hypothesis that SGK1 stimulates CFTR  $\text{Cl}^-$  currents by phosphorylating and inhibiting the ubiquitin ligase Nedd4-2, which inhibits the degradation of internalized CFTR as shown in Fig. 6.

Similar to what has been described for the regulation of ENaC (Debonneville et al. 2001, Snyder et al. 2002) and the cardiac Na channel SCN5A (Abriel et al., 2000), CFTR was down-regulated by Nedd4-2, presumably by ubiquitination of the transport channel protein. This effect was reversed by SGK1. As shown before (Debonneville et al. 2001, Snyder et al. 2002), SGK1 phosphorylated Nedd4-2 and thus inactivated the ubiquitin ligase (Fig. 7). In view of the regulation of CFTR by SGK1, mechanisms influencing the expression and activity of SGK1 in the epithelial target cells could be expected to regulate the CFTR protein abundance in the apical cell membrane and, thus,  $\text{Cl}^-$  transport.

The present experiments disclose two completely novel mechanisms involved in the regulation of epithelial  $\text{Cl}^-$  transport systems, i.e., the regulation by Nedd4-2 and by the protein kinase SGK1. The kinase increases the CFTR activity and stimulates CFTR mediated  $\text{Cl}^-$  transport. As shown for Nedd4-2, the effect is reversed by additional co-expression of SGK1. The effect is at least partially due to stimulation of the insertion of the channel into the cell

membrane and delaying the endocytotic retrieval of CFTR Cl<sup>-</sup> channel.

## Conclusion

The major new findings in this study are that CFTR was regulated by the ubiquitin ligase Nedd4-2 indicating that ubiquitination was required for the down-regulation of the channel. Activation of SGK1 led to phosphorylation and thus, inhibition of Nedd4-2 binding to its target, leading to impaired clearance of CFTR channel protein from the cell membrane. Thus, stimulation of SGK1 enhanced Cl<sup>-</sup> transport by increasing channel protein abundance in the cell membrane. This novel mechanism may play a role in the regulation of Cl<sup>-</sup> transport by several hormones and cytokines.

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## Conflict of interest

The author has no conflict of interest to declare.

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