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Original Paper

SPAK Sensitive Regulation of the Epithelial Na^+ Channel ENaC

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Key Words
Epithelial Na^+ channels • Oocytes • Mice • Colon • WNK

Abstract
Background/Aims: The WNK-dependent STE20/SPS1-related proline/alanine-rich kinase SPAK participates in the regulation of NaCl and Na^+,K^+,$2Cl^-$ cotransport and thus renal salt excretion. The present study explored whether SPAK has similarly the potential to regulate the epithelial Na^+ channel (ENaC). ***Methods:*** ENaC was expressed in *Xenopus* oocytes with or without additional expression of wild type SPAK, constitutively active ^{T233E}SPAK, WNK insensitive ^{T233A}SPAK or catalytically inactive ^{D212A}SPAK, and ENaC activity estimated from amiloride (50 μM) sensitive current (I_{amil}) in dual electrode voltage clamp experiments. Moreover, Ussing chamber was employed to determine I_{amil} in colonic tissue from wild type mice (*spak*$^{wt/wt}$) and from gene targeted mice carrying WNK insensitive SPAK (*spak*$^{tg/tg}$). ***Results:*** I_{amil} was observed in ENaC-expressing oocytes, but not in water-injected oocytes. In ENaC expressing oocytes I_{amil} was significantly increased following coexpression of wild-type SPAK and ^{T233E}SPAK, but not following coexpression of ^{T233A}SPAK or ^{D212A}SPAK. Colonic I_{amil} was significantly higher in *spak*$^{wt/wt}$ than in *spak*$^{tg/tg}$ mice. ***Conclusion:*** SPAK has the potential to up-regulate ENaC.

Introduction

SPAK (SPS1-related proline/alanine-rich kinase) is a powerful regulator of renal salt excretion and blood pressure [1-3]. SPAK activity is regulated by WNK (with-no-K[Lys]) kinases [1, 4-7], which similarly impact on ion transport and blood pressure [8-12]. Along

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those lines mutations of genes encoding WNK kinases underly Gordon's syndrome, a genetic disease characterized by hypertension and hyperkalaemia [5, 6, 13, 14]. SPAK sensitive transporters include NaCl and $Na^+,K^+,2Cl^-$ cotransporters [4, 7, 8, 15-25]. Moreover, SPAK and/or the related oxidative stress-responsive kinase 1 (OSR1) modify the function of further transport molecules including Na^+ coupled phosphate transport [26, 27], and Na^+/H^+ exchanger [28]. These kinases may thus participate in the regulation of further epithelial transport processes.

The present study explored whether SPAK modifies the epithelial Na^+ channel ENaC [29]. To this end, cRNA encoding ENaC was injected into *Xenopus* oocytes with or without cRNA encoding wild-type, constitutively active, WNK1-insensitive or catalytically inactive SPAK. As SPAK is known to be highly expressed in the colon [30], Ussing chamber experiments have been performed to quantify ENaC activity in colonic epithelium isolated from gene targeted mice expressing SPAK resistant to activation by WNK (*spak*$^{tg/tg}$) and from mice expressing wild type SPAK (*spak*$^{wt/wt}$).

Materials and Methods

Constructs

Constructs encoding rat ENaC [31], wild-type SPAK, WNK1 insensitive inactive ^{T233A}SPAK, constitutively active ^{T233E}SPAK, and catalytically inactive ^{D212A}SPAK [7] as well as wild type Nedd4-2 [32] were used for generation of cRNA as described previously [33, 34]

Voltage clamp in Xenopus oocytes

Xenopus oocytes were prepared as previously described [35]. 1 ng cRNA encoding each subunit of ENAC (α,β,γ) and 10 ng cRNA encoding wild-type SPAK, ^{T233A}SPAK, ^{T233E}SPAK, or ^{D212A}SPAK were injected on the same day after oocyte preparation [26, 36]. The oocytes were maintained at 17°C in ND96-A solution containing (in mM): 88.5 NaCl, 2 KCl, 1 MgC12, 1.8 CaC12, 5 HEPES, tretracycline (Sigma, 0.11 mM), ciprofloxacin (Sigma, 4 lM), gentamycin (Refobacin, 0.2 mM), theophylin (Euphylong, 0.5 mM), and sodium pyruvate (Sigma, 5 mM). The pH was adjusted to 7.5 by addition of NaOH. The voltage clamp experiments were performed at room temperature 3 days after injection [37, 38]. Two-electrode voltage-clamp recordings were obtained at a holding potential of -80 mV. ENaC was determined from amiloride (50 μM)-sensitive current (I_{amil}). The data were filtered at 10 Hz and recorded with a Digidata A/D-D/A converter and Clampex 9.2 software for data acquisition and analysis (Axon Instruments) [39]. The control superfusate (ND96) contained (in mM): 93.5 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES, pH 7.4. The flow rate of the superfusion was approx. 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s [40, 41].

Ussing chamber experiments

All animal experiments were conducted according to the German law for the welfare of animals and according to the guidelines of the American Physiological Society and were approved by local authorities (Regierungspräsidium Tübingen). Experiments were performed using colonic segments from 16-week old female gene targeted mice expressing SPAK resistant to WNK-dependent activation (*spak*$^{tg/tg}$) and in mice expressing wild-type SPAK (*spak*$^{wt/wt}$) [27]. Prior to the experiments mice had free access to food (1314, Altromin, Heidenau, Germany) and water *ad libitum*, and were kept under constant humidity (55 ± 10%), temperature (22 ± 2ºC) and 12h light-dark cycle conditions.

Mice were fasted for 6 hours prior to experiments. ENaC activity was estimated from the amiloride-sensitive potential difference and current across the colonic epithelium. After removing the outer serosal and the muscular layer of late distal colon under a microscope, tissues were mounted onto a custom-made mini-Ussing chamber with an opening diameter of 0.99 mm and an opening area of 0.00769 cm^2. Transepithelial potential difference (V_{te}) was determined continuously and transepithelial resistance (R_{te}) estimated from the voltage deflections (ΔV_{te}) elicited by imposing rectangular test currents of 1 μA and 1.2 s duration at a rate of 8/min. R_{te} was calculated according to Ohm's law [35, 42]. The serosal and luminal perfusate

contained (in mM): 145 NaCl, 1 $MgCl_2$, 2.6 Ca-gluconate, 0.4 KH_2PO_4, 1.6 K_2HPO_4, 5 glucose. To assess ENaC mediated transport, 50 µM amiloride (Sigma, Taufkirchen; in DMSO) was added to the luminal perfusate.

Metabolic cages

Mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) and they were maintained on a standard diet and had free access to tap water before the experiment [28, 43]. They were allowed a two day habituation period. Subsequently, feces were collected daily for four days. Feces were collected in separated tubes in order to assure quantitative collection.

Determination of serum aldosterone as well as serum, urinary and fecal electrolyte concentrations

To collect blood specimen, animals were lightly anesthetized and about 50 - 200 µl of blood was collected into serum tubes by puncturing the retro-orbital plexus [28]. The serum aldosterone concentration was determined using a commercial ELISA kit (Alpha Diagnostics International, Texas; USA). Fecal dry weight was obtained by drying the collected sample at 80°C for three hours. The fecal samples were prepared for determination of Na^+ content by dissolving in nitric acid (0.75 M HNO_3) and 48 hours at 50°C with continuous shaking. The homogenized samples were centrifuged at 3,500 g for 10 min and 1 ml of the supernatants were again centrifuged at 10,000 g for 5 min. Aliquots from the second supernatants were diluted and the Na^+ content of the supernatant was determined by flame photometry. The measured electrolyte concentrations were calculated to obtain the fecal sodium excretion in µmol per g of feces excreted within 24 hours.

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes or colonic segments investigated. All voltage clamp experiments were repeated with at least 2-3 batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using ANOVA (Tukey test or Kruskal-Wallis test) or two-tailed unpaired t-test, as appropriate. Results with * $p < 0.05$ were considered statistically significant.

Results

The present study explored whether WNK-dependent STE20/SPS1-related proline/alanine-rich kinase SPAK modifies the activity of the epithelial Na^+ channel ENaC. To this end, cRNA encoding ENaC was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding wild-type SPAK. ENaC activity was estimated from the current (I_{amil}) generated by the ENaC inhibitor amiloride (50 µM) as determined utilizing dual electrode voltage clamp. As illustrated in Fig.1, I_{amil} was virtually absent in water-injected *Xenopus* oocytes, indicating that *Xenopus* oocytes do not express appreciably endogenous ENaC (Fig. 1). In contrast, amiloride generated a large current in *Xenopus* oocytes injected with cRNA encoding ENaC. The additional injection of cRNA encoding wild-type SPAK was followed by a significant increase of I_{amil} in ENaC-expressing *Xenopus* oocytes.

Further experiments addressed whether the effect of wild type SPAK is mimicked by SPAK mutants. To this end, cRNA encoding ENaC was injected into *Xenopus laevis* oocytes with or without additional injection of cRNA encoding constitutively active ^{T233E}SPAK, WNK-resistant ^{T233A}SPAK or catalytically inactive ^{D212A}SPAK. As illustrated in Fig. 2, the coexpression of constitutively active ^{T233E}SPAK was followed by a significant increase of I_{amil} in ENaC-expressing *Xenopus* oocytes. In contrast, the additional expression of WNK insensitive inactive ^{T233A}SPAK or catalytically inactive ^{D212A}SPAK did not modify I_{amil} in ENaC-expressing *Xenopus* oocytes (Fig. 2).

ENaC is downregulated by the ubiquitin ligase Nedd4-2 [29]. In order to test, whether SPAK is effective by preventing the effect of Nedd4-2 on ENaC activity, experiments were performed in ENaC expressing oocytes without or with coexpression of Nedd4-2 and/or wild type SPAK. As illustrated in Fig. 3, the amiloride sensitive current in ENaC expressing *Xenopus* oocytes was significantly decreased by coxpression of Nedd4-2. The coexpression of

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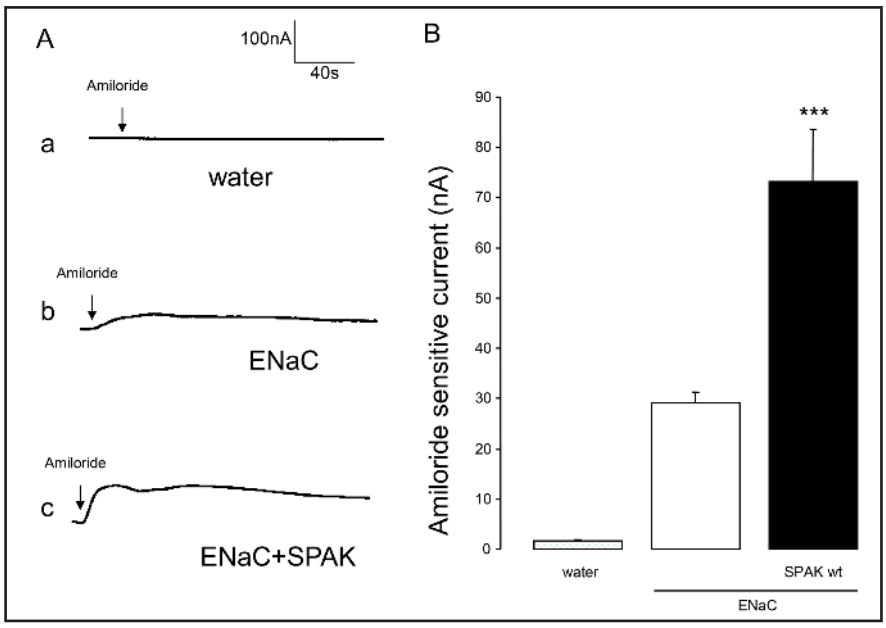

Fig. 1. Effect of wild-type SPAK on amiloride induced current in ENaC-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing amiloride (50 μM)-induced current (I_{amil}) at -80 mV holding potential in *Xenopus* oocytes injected with water (a), expressing ENaC alone (b), or expressing ENaC with additional coexpression of wild-type SPAK (c). B: Arithmetic means ± SEM (n = 3-12) of amiloride (50 μM)-induced current (I_{amil}) at -80 mV holding potential in *Xenopus* oocytes injected with water (dotted bar), expressing ENaC alone (white bar), or expressing ENaC together with wild-type SPAK (black bar). * **indicates statistically significant (p< 0.001) difference from *Xenopus* oocytes expressing ENaC alone (Tukey test).

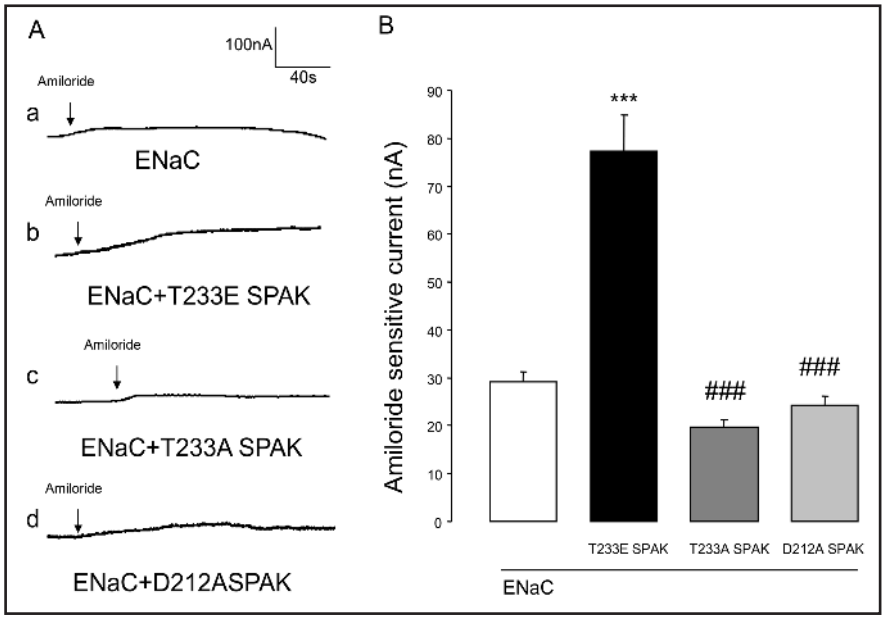

Fig. 2. Effect of constitutively active ^{T233E}SPAK, WNK insensitive inactive ^{T233A}SPAK or catalytically inactive ^{D212A}SPAK coexpression on amiloride induced current in ENaC-expressing *Xenopus laevis* oocytes. A: Representative original tracings showing amiloride (50 μM)-induced current (I_{amil}) at -80 mV holding potential in *Xenopus* oocytes expressing ENaC alone (a), or expressing ENaC with additional coexpression of constitutively active ^{T233E}SPAK (b), WNK insensitive ^{T233A}SPAK (c), or catalytically inactive ^{D212A}SPAK (d). B: Arithmetic means ± SEM (n = 10-12) of amiloride (50 μM)-induced current (I_{amil}) at -80 mV holding potential in *Xenopus* oocytes expressing ENaC alone (white bar), or expressing ENaC together with constitutively active ^{T233E}SPAK (black bar), WNK insensitive ^{T233A}SPAK (dark grey bar), or catalytically inactive ^{D212A}SPAK (light grey bar). ***indicates statistically significant (p < 0.001) difference from *Xenopus* oocytes expressing ENaC alone, ### (p<0.001) indicate statistically significant difference from the respective value with ^{T233E}SPAK expression alone (Tukey test).

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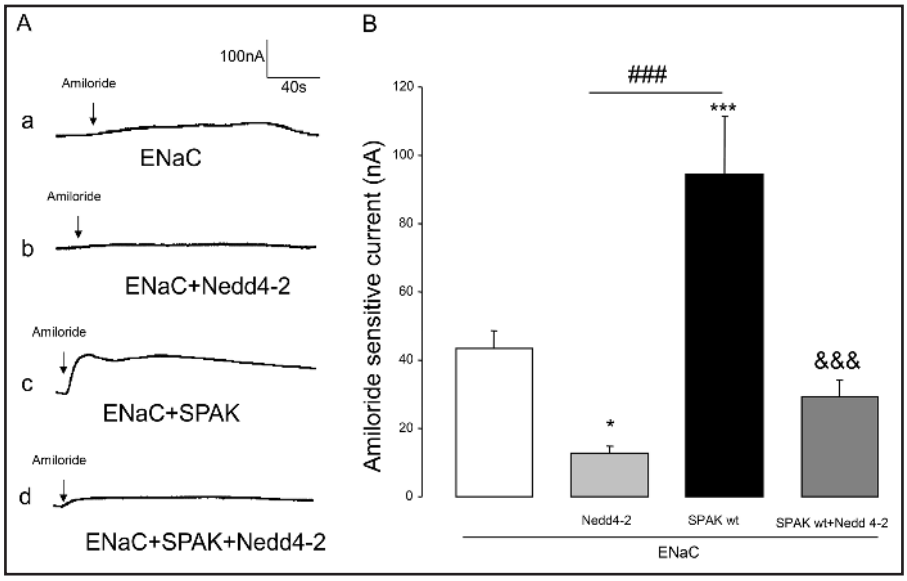

Fig. 3. Effect of Nedd4-2 in the presence and absence of SPAK on amiloride sensitive current in ENaC-expressing *Xenopus* oocytes. A. Original tracings of the amiloride (50 μM) sensitive current at -80 mV holding potential in *Xenopus* oocytes expressing ENaC either alone (a, ENaC), or with additional coexpression of Nedd4-2 (b, ENaC+Nedd4-2), SPAK (c, ENaC+ SPAK), or both SPAK and Nedd4-2 (d, ENaC+Nedd4-2+ SPAK). B. Arithmetic means ± SEM (n = 9-12) of the amiloride (50 μM) sensitive current at -80 mV holding potential in *Xenopus* oocytes expressing ENaC alone (ENaC, white bar), or expressing ENaC with additional coexpression of Nedd4-2 (light grey bar), of SPAK (black bar) or of SPAK and Nedd4-2 (dark grey bar). *** ($p<0.001$) indicate statistically significant difference from the value obtained in oocytes expressing ENaC alone, ### ($p<0.001$) indicate statistically significant difference from the respective value with Nedd4-2 expression alone, &&& ($p<0.001$) indicate statistically significant difference from the respective value with wild type SPAK expression alone (Tukey test).

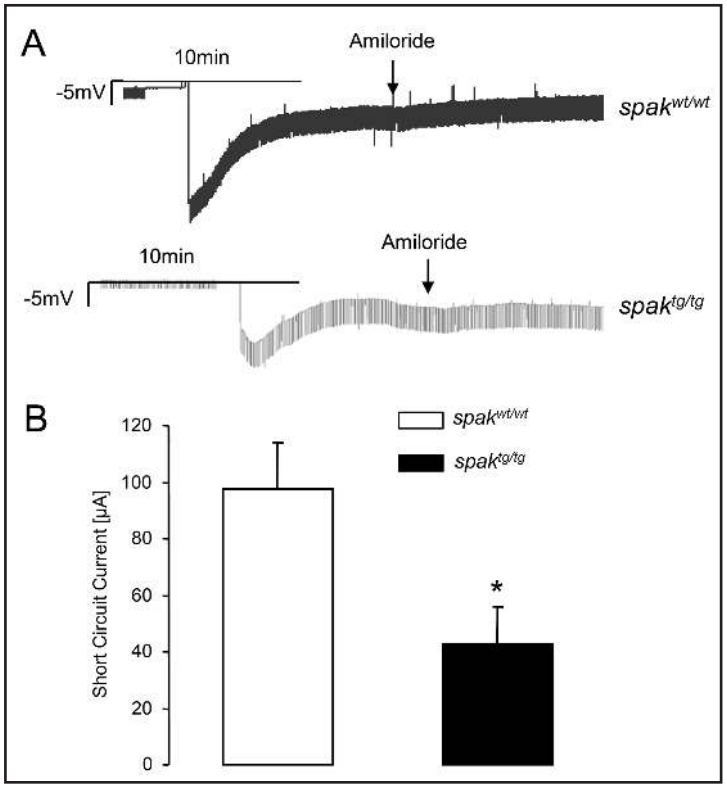

Fig. 4. Amiloride induced transepithelial current in colonic epithelia from $spak^{wt/wt}$and $spak^{tg/tg}$ mice. A. Original tracings illustrating the effect of test currents (1 μA) showing the effect of amiloride (50 μM) on the transepithelial colonic potential difference in $spak^{wt/wt}$ and $spak^{tg/tg}$ mice. Arrows highlight the addition of amiloride (50 μM). B. Arithmetic means ± SEM (n = 6) of the amiloride (50 μM) induced equivalent short-circuit current across colonic epithelium from $spak^{wt/wt}$ (white bar) and $spak^{tg/tg}$ mice (black bar). *($p<0.05$) indicates statistically significant difference from $spak^{wt/wt}$ (unpaired t-test).

Nedd4-2 similarly decreased the amiloride sensitive current in oocytes coexpressing ENaC and wild type SPAK.

In order to test whether upregulation of ENaC by SPAK plays a role *in vivo*, amiloride-induced current was measured in colonic epithelia utilizing Ussing chambers. As illustrated in Fig. 4, the colonic amiloride-induced current was significantly lower in gene targeted mice expressing WNK insensitive Spak ($spak^{tg/tg}$) than in wild-type littermates ($spak^{wt/wt}$). Fecal Na^+ concentration was similar in $spak^{tg/tg}$ mice (160 ± 4 µmol/g, n = 4) and in $spak^{wt/wt}$ mice (171 ± 3 µmol/g, n = 5). Similarly, plasma aldosterone levels were similar in $spak^{tg/tg}$ mice (207 ± 31 pg/dl, n = 4) and in $spak^{wt/wt}$ mice (254 ± 35 pg/dl, n = 4).

Discussion

The present study discloses a novel potential function of SPAK, i.e. stimulation of ENaC. Both, wild-type and constitutively active ^{T233E}SPAK, but not the catalytically inactive mutant ^{D212A}SPAK [7], upregulated ENaC activity. Thus, kinase activity is required for the stimulation of ENaC by SPAK. Moreover, the effect apparently requires WNK1 sensitive activation of SPAK, as the WNK insensitive ^{T233A}SPAK [7] was not capable to upregulate ENaC activity.

The present study did not address the cellular mechanisms involved in the SPAK sensitive regulation of ENaC. In theory, SPAK could be effective by phosphorylating ENaC itself or by modifying the functions of other kinases known to regulate ENaC. ENaC is regulated by a variety of kinases [44]. ENaC is stimulated by SGK isoforms, PKA, CK2, GRK2, IKKβ and PKD1 and inhibited by PKC, ERK1/2 and AMPK [44]. SGK is up-regulated by TORC2 and PDK1 [44]. The upregulation of ENaC following coexpression of SPAK could result from enhanced ENaC protein abundance in the cell membrane or activation of existing ENaC protein. Additional experiments are required to discriminate between those possibilities.

The functional significance of SPAK sensitive regulation of ENaC is illustrated by the observation that the amiloride sensitive current across the colonic epithelium is lower in mice carrying the WNK1 insensitive ^{T243A}SPAK ($spak^{tg/tg}$) than in wild-type SPAK ($spak^{wt/wt}$). In a previous study, the amiloride sensitive current across the colonic epithelium was shown to be higher in heterozygous mice carrying one allele of WNK1 resistant OSR1 [28]. Whether or not SPAK and OSR1 exert opposing functions in colonic epithelium, remains to be shown. Neither fecal Na^+ excretion nor aldosterone plasma levels were significantly different between $spak^{tg/tg}$ and $spak^{wt/wt}$ mice. Thus, the impact of SPAK resistance to WNK1 on renal salt excretion and extracellular fluid volume is apparently small and/or compensated by other mechanisms regulating fecal salt excretion and extracellular fluid volume.

Dysregulation of ENaC may affect a variety of functions including deranged regulation of renal salt excretion [45], blood pressure [29], cell volume [46], fluid transport in the lung [47, 48], endothelial function [49] and embryo implantation [50]. To which extent SPAK or WNK sensitive activation of SPAK participates in the regulation of those functions remains to be established.

Conclusion

In conclusion, SPAK has the potential to up-regulate ENaC and may thus contribute to the complex regulatory network of this important channel.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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