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# SPAK and OSR1 Kinases Bind and Phosphorylate the $\beta_2$ -Adrenergic Receptor

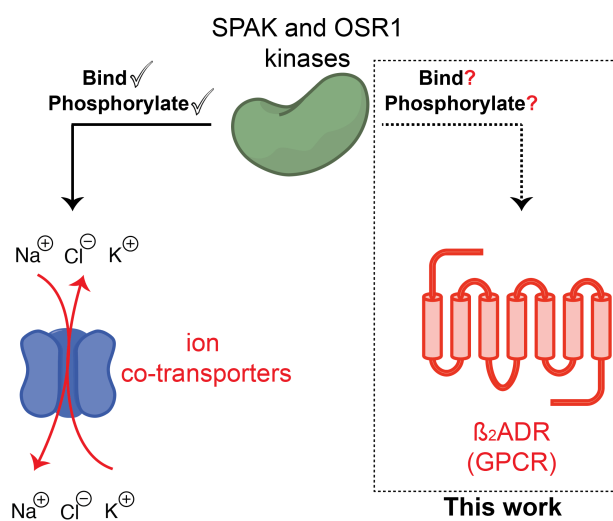
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SPAK, OSR1,  $\beta_2$ ADR, Binding, Phosphorylation.

## TOC



## ABSTRACT

SPAK and OSR1 are two cytoplasmic serine/threonine protein kinases that regulate the function of a series of sodium, potassium and chloride co-transporters via phosphorylation. Over recent years, it has emerged that these two kinases may have diverse function beyond the regulation of ion co-transporters. Inspired by this, we explored whether SPAK and OSR1 kinases impact physically and phosphorylate the  $\beta_2$ -adrenergic receptor ( $\beta_2$ ADR). Herein, we report that the amino acid sequence of the human  $\beta_2$ ADR displays a SPAK/OSR1 consensus binding motif and using a series of pulldown and *in vitro* kinase assays we show that SPAK and OSR1 bind the  $\beta_2$ ADR and phosphorylate it *in vitro*. This work provides a notable example of SPAK and OSR1 kinases binding to a G-protein coupled receptor and taps into the potential of these protein kinases in regulating membrane receptors beyond ion co-transporters.

## 1. INTRODUCTION

The with-no-lysine (WNK) serine/threonine protein kinases 1-4 are key regulators of ion homeostasis [1-3]. Mutations in the genes that encode these kinases cause an inherited form of hypertension known as Gordon's syndrome (**Fig. 1a**) [4]. The genetic link between WNK-signalling and hypertension was further strengthened in 2012 when it was reported[5, 6] that mutations in two ubiquitin E3 ligases known as kelch-like 3 (KLHL3) and Cullin 3 (Cul3), which we[7] and others[8, 9] later found to control the activity of WNK kinases, resulted in hypertension (**Fig. 1a**).

WNK kinases bind to and phosphorylate two serine/threonine protein kinases termed Ste20p-related proline alanine-rich kinase (SPAK) and oxidant stress-responsive protein kinase 1 (OSR1), [10] which in complex[11] with the scaffolding mouse only protein 25 (MO25) phosphorylate a plethora of sodium, potassium and chloride ion co-transporters such as the Na<sup>+</sup>-K<sup>+</sup>-Cl<sup>-</sup> co-transporters 1 and 2 (NKCC1 and NKCC2, respectively) and the Na<sup>+</sup>-Cl<sup>-</sup> co-transporter (NCC) (**Fig. 1a**) [10].

To date, most studies on the WNK-SPAK/OSR1 signalling cascade have focused on its role in regulating blood pressure through the phosphorylation of ion co-transporters [1, 2]. In 2011, however, the transcription of the WNK4 gene in the kidney was reported to be regulated by the  $\beta_2$ ADR[12] though some of these findings were later contested [13]. In 2013, a study from the Kobilka lab where the  $\beta_2$ ADR was pulled down from bovine heart lysates treated with either the inverse agonist carazolol or the full agonist BI-167107 followed by proteomics identified ca. 250 endogenous proteins as possible binders to the  $\beta_2$ ADR among which was the protein kinase SPAK [14]. Collectively, these studies provided an indication of a possible novel and unexplored crosstalk between the WNK-signalling pathway and the  $\beta_2$ ADR. Intrigued by these studies, we embarked on verifying whether SPAK and OSR1 kinases bind and phosphorylate the  $\beta_2$ ADR.

## 2. MATERIALS AND METHODS

**2.1. Materials.** The synthetic biotinylated 18-mer RFQV containing peptide (biotin-C<sub>6</sub>-SEEGKPQLVGRFQVTSSK, catalogue number; 331938), AFQV containing peptide (biotin-C<sub>6</sub>-SEEGKPQLVGAFQVTSSK, catalogue number: 372447), RFHV containing peptide (biotin-C<sub>6</sub>-DKSEGRFHVQNLSQ, catalogue number: 372432), and AFHV containing peptide (biotin-C<sub>6</sub>-DKSEGAHFHVQNLSQ, catalogue number: 372438) as well as CATCHtide (RRHYYYDTHNTNTYYLR-TFGHNTRR) were purchased from GLS Peptide synthesis, Shanghai, China. The cDNA plasmids pCMV5-GST-EV (DU41864), pEBG-HA- SPAK (DU2923), pCMV5-FLAG-NKCC2-FL-WT (DU10098), pcDNA5-FRT/TO- FLAG-NKCC2-R20A (DU17023), pCMV-FLAG-NKCC2 (1-174) WT (DU13857), pCMV5-FLAG-β<sub>2</sub>AR-R239A (DU39345), pEBG-HA-OSR1T185E (DU6130), pGEX- NKCC2 (1-174) (DU13763), pGEX-OSR1-T185E full length (DU 6231) and pGEX- MO25α (DU2945) were purchased from the MRC PPU Reagents and Services (Dundee). pcDNA5-FRT/TO-FLAG-β<sub>2</sub>AR-WT (lot:390869S) was purchased from GenScript.

**2.2. Buffers.** Lysis buffer 1 (LB1). *Cell lysis buffer:* 50 mM Tris/HCl (pH 7.5), 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM EDTA, 50 mM sodium fluoride, 0.27 M sucrose, 5 mM sodium pyrophosphate, 1 % (w/v) Nonidet P40 plus protease inhibitors 0.1 mM phenylmethanesulfonylfluoride (PMSF), 1 mM benzamidine, and 0.1 % (v/v) 2-mercaptoethanol. Lysis buffer 2 (LB2) was LB1 supplemented with 1 % (w/v) *n*-dodecyl β-D-maltoside (catalogue number; 89902, ThermoFisher). Lysis buffer 3 (LB3) was used to solubilize β<sub>2</sub>AR-transfected mammalian cells and it contained 50 mM HEPES (pH 7.5), 0.5 % Nonidet P40, 145 mM sodium chloride, 2 mM EDTA, and 10 % (v/v) glycerol, 0.1 mM PMSF, 1 mM benzamidine, 0.1 % (v/v) 2-mercaptoethanol and 1 % (w/v) final concentration of *n*-dodecyl β-D-maltoside was freshly added before cell lysing. Buffer A:[11] 50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA and 0.1% (v/v) 2-mercaptoethanol.

**2.3. Cell culture.** Mammalian cells were cultured in T-75 flasks, 10-cm or 6-well plates. The cells were cultured in DMEM supplemented with 10 % (v/v) FBS and 1 % (v/v) Pen/Strep. Once they reached 80-90 % confluency (log phase), the cells were regularly passaged in 1:5 ratio every 2-3 days. The cells were washed once by 3-5 ml of PBS after the removal of the media. Then, they were detached with 2 ml trypsin. Typically, the cells were grown in 12-15 ml media in T75, 7 ml media in 10-cm dishes or 3 ml media in the 6-well plates. All of the flasks and plates were maintained at 5 % CO<sub>2</sub> humidified incubator at 37 °C.

For protein expression in mammalian cells, HEK293 cells (passage range 5-25) were plated either in 6-well plates or 10-cm dishes. Once the cells reached 40-50 % confluency, they were transfected using the PEI method as reported previously[10]. 1 µg of cDNA clones with 2 µl of 1 mg/ml PEI was mixed in a ratio of 1:2 in 200 µl FBS-free DMEM media per well of 6-well plates. 3 µg cDNA with 20 µl 1 mg/ml PEI in 1 ml FBS- free DMEM media for a single 10-cm dish. Then, the mixture was vortexed gently and left at room temperature for 25 min. This mixture was then added to the cells carefully to avoid their detachment and the cells were incubated at 37 °C. 36-48 hrs post-transfection, the cells were harvested, or underwent treatment as needed.

**2.4. Preparation of mouse heart samples.** The mouse heart samples were prepared as previously reported [15].

### **2.5. Pulldown and Immunoprecipitation Protocols.**

#### *2.5.1. Biotin-RFxV pulldown of SPAK and OSR1*

Once HEK293 cells cultured in 10-cm dishes were 80-90 % confluent, they were harvested and lysed. Then 1-5 mg of the total cell lysates was incubated with 3 µg of either the RFQV-peptide, the RFHV-peptide, the AFQV-peptide or the AFHV- peptide for 15 min at 4 °C on rotator shaker. After incubation, 40 µl of streptavidin 50 % slurry (which had been washed and equilibrated in lysis buffer) was added to each sample. The samples were then incubated on

rotator shaker for 15 min at 4 °C.[16] After that, the beads were collected by centrifuging at 1,000 g for 2 min at 4 °C. The beads were subsequently washed twice with 1 ml LB1 and washed again twice with 1 ml buffer A. Finally, the beads were prepared for SDS-PAGE as described below.

### 2.5.2. GST affinity pull down

Once cultured HEK293 cells in 10-cm dishes reached 50 % confluency, they were transfected with glutathione S-transferase (GST)-tagged proteins e.g. SPAK and OSR1 alone, or co-transfected with other FLAG-fusion proteins. 36 hrs post-transfections, the cells were harvested by adding 0.4 ml of suitable lysis buffer. Then, the cell suspensions were collected and clarified from cellular debris by centrifugation at 12,000 g for 10 min at 4 °C. The supernatants were collected, and protein concentration was measured by the Bradford assay and the samples were prepared for SDS-PAGE to assess protein expression while the remaining lysates were stored at -80 °C.

Pulldown of GST-tagged proteins was carried out by using glutathione Sepharose high performance (GST) beads (catalogue number: 17-527-01, GE Healthcare). GST beads were washed with 1 ml PBS three times by incubating with PBS and then centrifuging at 1,000 g at 4 °C for 5 min. After this, the collected GST- beads equilibrated in PBS at 50 % slurry. 0.5 mg of the cell lysates was incubated on rotator shaker in a 20 µl slurry of GST-beads either overnight at 4 °C or 1 hr at room temperature.[16] The beads were then collected by centrifugation at 1,000 g at 4 °C for 5 min. The collected beads were washed twice with 1 ml LB1 and twice with 1 ml buffer A. Finally, the beads were prepared for SDS-PAGE electrophoresis as described below.

### 2.5.3. Affinity pulldown of FLAG-β<sub>2</sub>AR

HEK293 cells were cultured and transiently transfected in 10-cm plates using PEI. 36-48 hrs post-transfection, the cells were washed once with 2 ml ice-cold PBS and detached by adding 400  $\mu$ l/plate of ice cold LB3. Following cell detachment by scrappers, the suspension was vortexed and incubated on rotator shaker for 2 hrs or overnight at 4 °C.[15] The lysate was subsequently centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was collected, and samples were prepared for SDS-PAGE to check receptor expression while the remaining lysate was stored at -80 °C.

In order to isolate FLAG- $\beta_2$ AR from cell lysate, pre-clearing of the cell lysate was first performed three times by incubating 1 mg cell lysate with 100  $\mu$ l of 50 % slurry of protein-G-Sepharose beads (GE Health care life sciences, catalogue number: PG5- 161501) on rotator shaker at 4 °C for 10 min. After this, the beads were collected by centrifuging at 1,000 g at 4 °C for 5 min. Anti-FLAG antibody (F3165, Sigma) conjugated to the protein-G Sepharose beads was added to the pre-cleared lysate at a ratio of 1:100, (10  $\mu$ g conjugated-antibody: 1 mg cell lysate).[10] After incubation for 1 hr on rotator shaker at 4 °C, the solid beads were collected by centrifuging at 1,000 g for 5 min at 4 °C. The beads were then washed twice by resuspending in 1 ml of LB3, centrifuging at 1,000 g for 5 min at 4 °C. For preparing samples for kinase assay, beads were washed two more washes with LB3 contained 0.5 M NaCl. After the last spin the beads were subsequently washed twice by resuspending in 1 ml buffer A and centrifuging at 1,000 g for 5 min at 4 °C. For Western blotting, proteins were eluted from the beads, by adding 60-80  $\mu$ l of 1x SDS-sample buffer, mixed well by a bench vertex and heated in a dry heater at 90 °C for 10 min. Then samples were stored at -20 °C.

#### 2.5.4. Immunoprecipitation of endogenous OSR1/SPAK

To immunoprecipitate endogenous SPAK from the mouse heart, pre-clearing steps were carried out as before. Then, 50  $\mu$ g of conjugated anti- OSR1 or anti-SPAK antibody was added to 5 mg of total protein lysate [10]. After incubation for 1 hr on rotator shaker at 4 °C, the beads



were collected by centrifuging at 1,000 g for 5 min at 4 °C. The beads were washed twice by resuspending in 1 ml LB1, then centrifuging at 1,000 g for 5 min at 4 °C. After the last spin, the beads were washed twice by resuspending in 1 ml buffer A and centrifuging at 1,000 g for 5 min at 4 °C. Then, 60 µl of 1x SDS-sample buffer was added to the beads and mixed well by bench vortex. Subsequently, the samples were either prepared for SDS-PAGE as before or stored at -20 °C.

**2.6. SDS-PAGE electrophoresis and Western blotting.** These were conducted using the protocols that were reported by Filippi B.M. *et al.* 2011[11].

**2.7. ADP-Glo™ In Vitro Kinase Assay.** OSR1-T185E, MO25 $\alpha$  and NKCC2 (1-174) were expressed and purified from LB21 *E.coli* as reported previously[11] while FLAG- $\beta_2$ AR was immunoprecipitated from transfected HEK293 cells. Non-radioactive ATP was used in this assay. The kinase reaction was performed in 1.5 ml Eppendorf tubes at a final volume of 25 µl/reaction in triplicate. Each reaction contained 0.4 µM OSR1-T185E with the presence or absence of 2 µM MO25 $\alpha$ . The substrates were either 250 µM CATCHtide (RRHYYYDTHNTNTYYLRTFGHNTRR), 5 µM GST-cleaved NKCC2 (1-174) that were used as positive control, or 10 µg beads conjugated to immunoprecipitated  $\beta_2$ AR. After all proteins were freshly prepared in kinase buffer which containing 10 mM MgCl<sub>2</sub>, and 0.1 mM ATP.[17] The samples were incubated on 30 °C for 40 min on gentle agitation. Then, the samples were transferred into a 96-wells plate and developed according manufacturer's instructions (ADP-Glo™ kit, Promega). This involved adding 25 µl of reagent-1 to each sample and incubation for 40 min at room temperature. Then, 50 µl of reagent-2 was added to each sample followed by incubation for 1 hr at room temperature.[18] The luminescence was subsequently measured by reading the plate on BMG FLUOStar Omega plate reader. The results were analysed by Graph Pad Prism and the data was presented as mean  $\pm$  SD of three independent experiments.

### 3. RESULTS AND DISCUSSION

The binding of SPAK and OSR1 kinases to their upstream kinases, WNKs, and downstream ion co-transporters is mediated by their highly conserved C-terminal domains, which bind unique Arginine-Phenylalanine-any amino acid-Valine/Isoleucine (RFx[V/I]) tetrapeptides from the upstream and downstream protein binders [19]. With this in mind, we first analysed the amino acid sequence of the human  $\beta$ ADRs 1-3 to determine if they have the SPAK/OSR1 binding motif. Such analysis showed that the human  $\beta_2$ ADR possesses an RFHV motif (239-242), which matches perfectly the SPAK/OSR1 RFx[V/I] consensus binding motif, while human  $\beta_1$  and  $\beta_3$ ADRs did not (**Fig. 1b**). Previous work focused on determining the 3D structure of the human  $\beta_2$ ADR indicated that the RFHV motif is located in the cytoplasmic loop (loop 3) of the  $\beta_2$ ADR [20], thus supporting its possible binding to cytoplasmic proteins. To establish whether the  $\beta_2$ ADR's RFHV motif mediates binding to SPAK and OSR1 kinases, we synthesised an *N*-terminally biotinylated 14-mer peptide derived from the human  $\beta_2$ ADR (aa. 234-248), which contains the RFHV motif. In addition, we also synthesised the same peptide with one single point mutation where the arginine (R) of the RFHV motif was mutated to alanine to generate the corresponding ARHV 14-mer peptide. As controls, we used an 18-mer RFQV peptide derived from WNK4 and its single point mutant 18-mer AFQV peptide [16]. These peptides were then incubated individually with HEK293 lysates, which endogenously express SPAK and OSR1 kinases. This was then followed by a biotin-streptavidin pulldown assay and the results showed that the positive control RFQV peptide pulled down endogenous SPAK while its corresponding AFQV did not as expected (**Fig. 2a**) [16]. Encouragingly, the  $\beta_2$ ADR-derived RFHV peptide pulled down endogenous SPAK while its corresponding AFHV mutant did not (**Fig. 2a**). This suggested that this  $\beta_2$ ADR-derived motif mediated the binding to SPAK. Next, we overexpressed full-length GST-tagged SPAK wild-type (WT) and FLAG-tagged  $\beta_2$ ADR in HEK293 cells. GST-affinity pulldown of the

overexpressed SPAK resulted in a clear pulldown of the overexpressed FLAG-tagged  $\beta_2$ ADR as shown by the FLAG blot (**Fig. 2b**). Interestingly, the addition of the synthetic SPAK-derived 18-mer RFQV peptide to the cell lysates resulted in competing out FLAG-  $\beta_2$ ADR binding to GST-SPAK (**Fig. 2b**). GST-pulldown from untransfected cells and the GST empty vector cells did not show any detectable FLAG-  $\beta_2$ ADR binding (**Fig. 2b**). To investigate this further, in HEK293 we overexpressed a fragment of FLAG-tagged NKCC2 WT, a physiological substrate of SPAK and OSR1 kinases, and its R20A mutant, which is known not to bind SPAK and OSR1 [16]. In parallel, we also overexpressed full-length FLAG-tagged  $\beta_2$ ADR WT and its R239A mutant, which corresponds to the AFHV mutant peptide. Following GST-pulldown, we performed Western blotting for FLAG. As expected, NKCC2 WT was pulled down while the binding to its R20A mutant led to significant reduction of binding to GST-SPAK (**Fig. 2c**). Encouragingly, GST-SPAK pulldown resulted in pulling down FLAG-  $\beta_2$ ADR WT, but not its R239A mutant (**Fig. 2c**). The GST and FLAG blots of the total lysate confirmed the overexpression of SPAK, NKCC2 and  $\beta_2$ ADR WT and mutants in equal amounts (**Fig. 2c**).

To establish if the  $\beta_2$ ADR binds SPAK and OSR1 when expressed at endogenous levels in native tissue, endogenous SPAK was immunoprecipitated from mouse heart lysates. To maximise the extraction of the  $\beta_2$ ADR from the membrane, we used two different mild detergents in lysing the mouse heart tissues; NP40 and NP40 plus dodecyl  $\beta$ -D-maltoside (D $\beta$ M), a combination that was used previously with success in immunoprecipitating  $\beta_2$ ADR[15]. The results showed that the immunoprecipitation of endogenous SPAK pulled-down endogenous  $\beta_2$ ADR (**Fig. 2d**) and this was more prominent in the D $\beta$ M-containing lysis buffer. Critically, the empty beads did not show any pull down of  $\beta_2$ ADR (**Fig. 2d**). This provided a compelling evidence of the binding of SPAK and the  $\beta_2$ ADR at the endogenous level.

Once the binding of SPAK to the  $\beta_2$ ADR was confirmed, we then explored whether SPAK and OSR1 kinases phosphorylate the  $\beta_2$ ADR *in vitro*. First, OSR1 T185E, where the threonine was swapped into a glutamic acid to mimic phosphorylation and generate a constitutively active OSR1,[10] was used to phosphorylate FLAG- $\beta_2$ ADR WT and GST-NKCC2 (1-174) (as a positive control). The results showed that OSR1 T185E was able to phosphorylate GST-NKCC2 *in vitro* as expected and this was suppressed by verteporfin, an OSR1 inhibitor[17] (**Fig. 3a**). Interestingly, OSR1 T185E was also able to phosphorylate FLAG- $\beta_2$ ADR WT to the same extent as the known substrate NKCC2 (**Fig. 3a**). Notably, the *in vitro* phosphorylation of the FLAG- $\beta_2$ ADR WT by OSR1 T185E was also suppressed by the OSR1 inhibitor verteporfin (**Fig. 3A**). Using the same kinase assay and employing the FLAG- $\beta_2$ ADR R239A protein as a substrate led to a significant reduction in its phosphorylation by OSR1 T185E as compared to that observed with FLAG- $\beta_2$ ADR WT (**Fig. 3b**).

In conclusion, this work provides evidence of SPAK and OSR1 kinases binding to the  $\beta_2$ ADR and that these kinases phosphorylate *in vitro* this pharmacologically important G-protein coupled receptor. The results presented herein will stimulate further research into mapping the exact phosphorylation sites of the  $\beta_2$ ADR that are phosphorylated by SPAK and OSR1 kinases as well as efforts aimed at unravelling the physiological relevance of such phosphorylation. Additionally, they suggest that SPAK and OSR1 kinases are likely to bind and regulate the function of other membrane receptors beyond ion co-transporters, a possibility that is currently unexplored.

### **Declaration of Competing Interests**

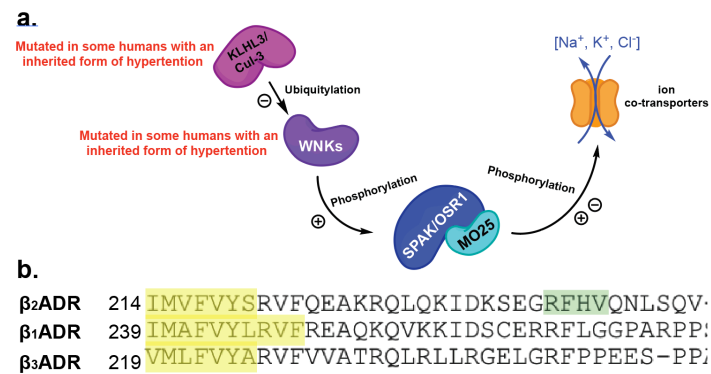
The authors declare no competing financial interest.

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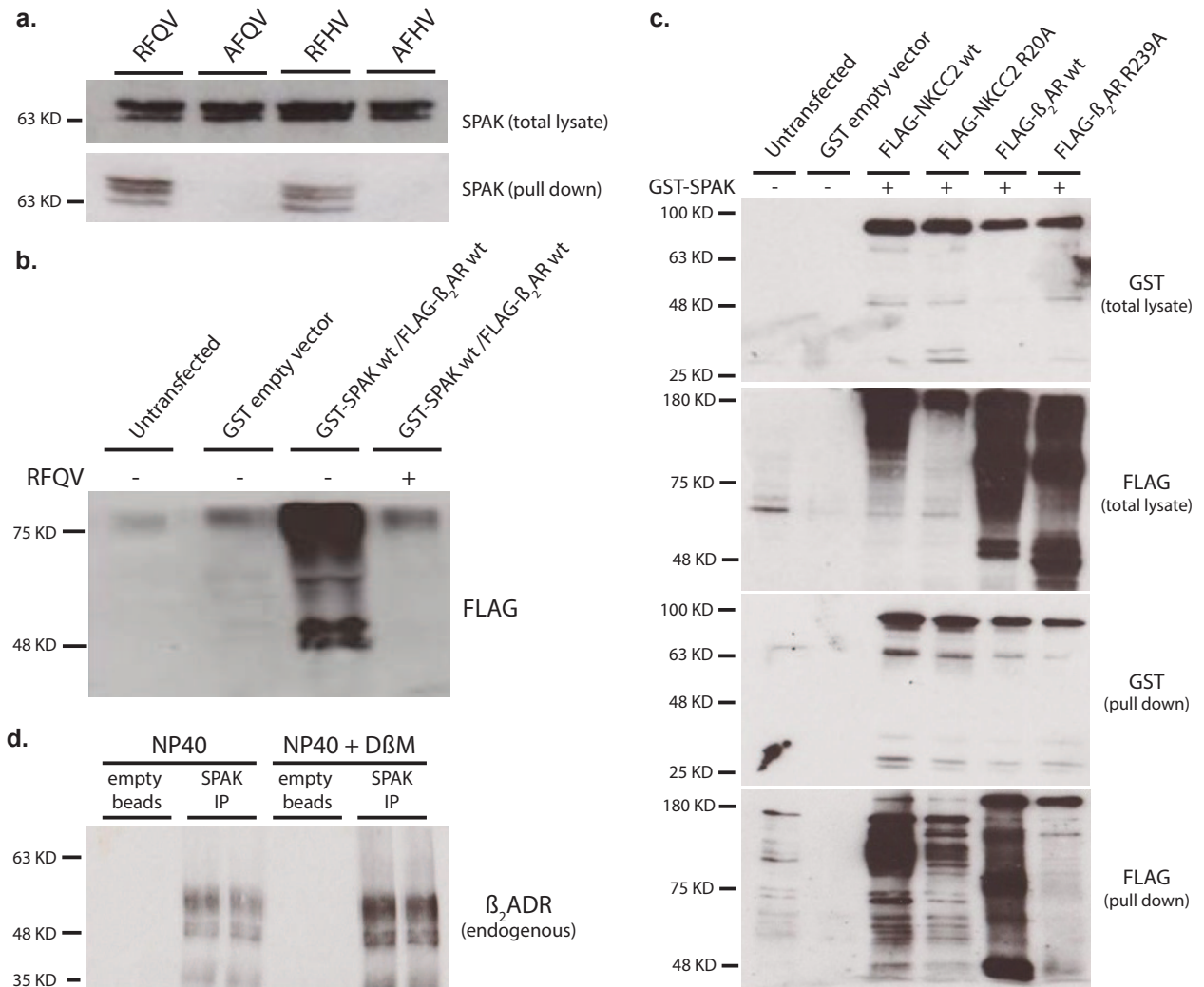
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## FIGURES



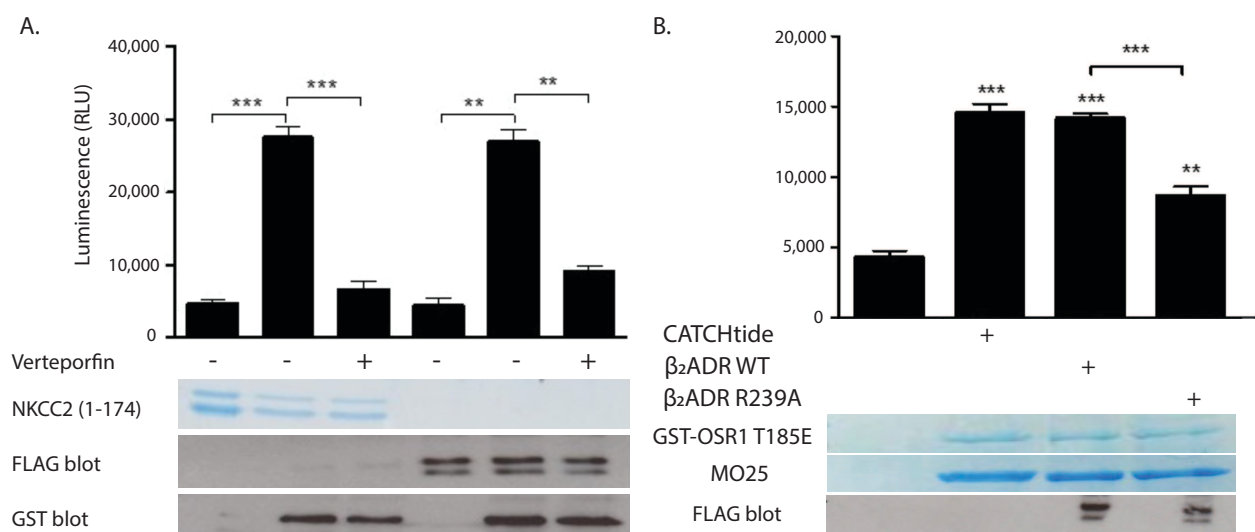
**Figure 1. a.** A representation of the main components of the WNK-SPAK/OSR1 signalling pathway. WNK kinases phosphorylate SPAK and OSR1, which in complex with the scaffolding protein MO25, phosphorylate a series ion co-transporters such as NKCC1/2, NCC and KCC. (-) indicates inhibition while (+) indicates activation. **b.** Sequence alignment of the human  $\beta$ ADR 1-3 showing the region of the  $\beta_2$ ADR with the possible SPAK/OSR1 RFHV binding motif (shaded in green). The yellow shaded regions are transmembrane sequences. The sequence alignment was conducted using UniProt Align.



**Figure 2. SPAK and OSR1 kinases bind the  $\beta_2$ ADR.** **a.** 5 mg of untransfected HEK293 lysate was incubated with 3  $\mu$ g of 1) the 18-mer RFQV peptide (SEEGKPQLVGRRFQVTSSK equivalent to human SPAK amino acids 379-394), which bind SPAK and OSR1, 2) its single point mutant 18-mer AFQV peptide (SEEGKPQLVGAAFQVTSSK equivalent to human SPAK amino acids 379-394), which does not bind SPAK and OSR1, 3) 14-mer RFHV peptide (DKSEGRRFHVQNLSQ) derived from the human  $\beta_2$ ADR (amino acids aa. 239-242) and 4) its corresponding mutant single point mutant 14-mer AFHV peptide (DKSEGAAFHVQNLSQ). All of these peptides carry an *N*-terminal biotin. Following streptavidin-biotin, pull down the material underwent western blotting for endogenous SPAK. *n* = 3. **b.** HEK293 were co-transfected with GST-SPAK full length and FLAG- $\beta_2$ ADR. The cells were lysed 48 h post-



transfection and the lysates were incubated with the 18-mer RFQV peptide for 2 h at 4 °C where indicated. Following GST-pull down, the pulled down material was blotted for FLAG to check FLAG- $\beta_2$ ADR pull down. **c.** HEK293 cells were transiently transfected with GST empty vector or co-transfected with GST-SPAK wildtype and either with Flag-NKCC2 full-length wildtype or its mutant R20A, or FLAG-  $\beta_2$ ADR wildtype or FLAG- $\beta_2$ ADR R239A. After GST pulldown, the samples underwent western blotting for FLAG. **d.** Mouse heart tissues were lysed in either a lysis buffer containing the detergent NP40 or a lysis buffer with NP40 supplemented with D $\beta$ M. Endogenous SPAK was subsequently immunoprecipitated from both samples and the obtained material underwent Western blotting for SPAK.



**Figure 3. OSR1 phosphorylates  $\beta_2$ ADR *in vitro*.** **a.** OSR1 T185E, where T185 was mutated into glutamic acid to mimic phosphorylation and generate a constitutively active OSR1,[11] was used to phosphorylate FLAG- $\beta_2$ ADR WT and GST-NKCC2 (1-174) in the presence or absence of the OSR1 inhibitor Verteporfin. **b.** Phosphorylation of FLAG- $\beta_2$ ADR WT and its R239A mutant by OSR1 T185E *in vitro*. Phosphorylation of the OSR1 peptide substrate CATCHtide[16] was used as a control. FLAG and GST blots were used to show the loading of

the  $\beta_2$ ADR WT, its R239 mutant and the NKCC2 (1-174). The FLAG- $\beta_2$ ADR WT and R239A were pulled down from 200  $\mu$ g of HEK293 total lysates overexpressing the FLAG-tagged protein using 10  $\mu$ g of FLAG antibody conjugated to sepharose beads. GST-NKCC2 (1-174) was purified from *E.coli* as reported,[21] and the *in vitro* kinase assay was conducted using Promega's ADP-Glo as we reported previously.[18] All results are represented as mean  $\pm$  S.E.M for triplicate samples, *P* value was calculated by one-way ANOVA, *P* < 0.05 was determined as statistical significance. (\*\*): *P* value = 0.0001 and (\*\*): *P* value =0.0025.