The Cul4-DDB1-WDR3/WDR6 Complex Binds SPAK and OSR1 Kinases in a Phosphorylation-Dependent Manner

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Abstract: SPAK and OSR1 are two protein kinases that play critical roles in regulating ion homeostasis. They are activated under osmotic stress via phosphorylation by their upstream WNK kinases at a conserved threonine site on their *T*-loops. Additionally, WNK kinases phosphorylate SPAK and OSR1 at a highly conserved serine residue on their *S*-motif, the function of which remains elusive. Using affinity pull down and mass spectrometry, we identified the E3 ubiquitin ligase complex Cullin 4-DDB1-WDR3/WDR6 as a binder to OSR1 kinase in a SPAK/OSR1 *S*-motif phosphorylation-dependent manner. This binding was found to be compromised by *S*-motif phosphorylation following osmotic stress. Using proteasomal and neddylation inhibitors, we subsequently showed the that OSR1 ubiquitylation was abolished under osmotic stress when its *S*-motif is phosphorylated. These results provide the first example of an E3 ubiquitin ligase system that binds the OSR1 kinase and, thus, links the CRL4 complex to ion homeostasis.

The STE20/SPS1-related kinase (SPAK) and the Oxidative-Stress-Responsive kinase 1 (OSR1) are two cytoplasmic serine/threonine protein kinases that phosphorylate a myriad of sodium, potassium and chloride ion co-transporters such as the sodium (Na), potassium (K) and chloride co-transporters 1 and 2 (NKCC1 and NKCC2), the potassium (K) chloride (Cl) co-transporters (KCCs) and the sodium (Na) and chloride (Cl) cotransporter (NCC) (**Figure 1A**).[1] Human SPAK and OSR1 kinases are 68% identical in sequence and share a highly conserved 92-residue *C*-terminal domain (residues 456–547 for SPAK and 436–527 for OSR1), which are 79% identical in sequence.[2] Although under normal conditions these kinases are inactive, they become activated by osmotic stress.[3]

At the molecular level, SPAK and OSR1 activation is mediated by *T*-loop phosphorylation at a highly conserved threonine residue (T233 for SPAK and T185 for OSR1) by With No lysine kinases (WNKs) (**Figure 1A**).[4] WNK kinases are a family of serine/threonine protein kinases that are mutated in some individuals with an inherited form of hypertension known as Gordon's syndrome.[5] Notably, WNK-phosphorylation of SPAK and OSR1 kinases at their *T*-loops does not lead to their maximal catalytic activity as for this to be achieved, they need to subsequently bind a scaffolding protein known as Mouse Only protein 25 (MO25).[6] In humans, there are two isoforms of MO25, α and β,[7] and they both activate WNK-phosphorylated OSR1 and SPAK kinases to a similar level.[6]

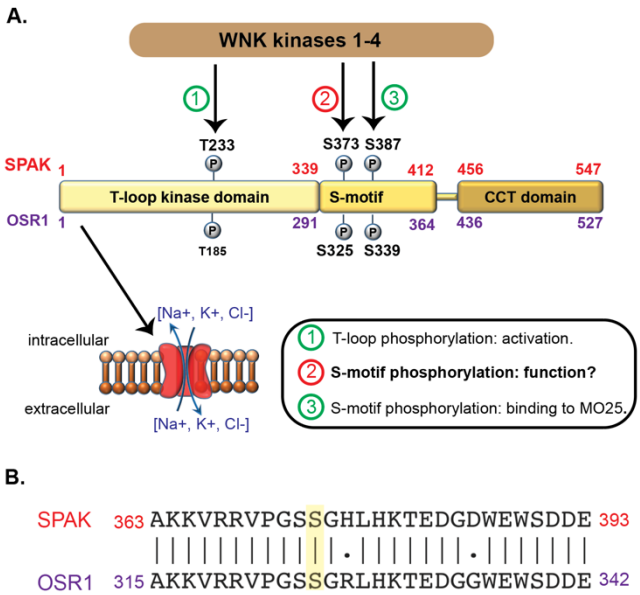

Figure 1. The WNK-SPAK/OSR1 signaling cascade. A. WNK kinases phosphorylate SPAK and OSR1 kinases at numerous sites including that in the T-loop and those in the S-motif (1-3). The function of these phosphorylation are well established apart from the *S*-motif phosphorylation (2). Once phosphorylated by WNK kinases, SPAK and OSR1 kinases phosphorylate a series of sodium, potassium and chloride ion co-transporters. Solid line arrows indicate phosphorylation. B. Sequence alignemnt of the human SPAK and OSR1 protein sequences showing the highly conserved region around the SPAK S373 and OSR1 S325 WNK phosphorylation sites (highlighted in yellow). Sequence alignemnt was conducted using EMBL-EBI EMBOSS Matcher.

Beyond WNK-phosphorylation of SPAK and OSR1 at their *T*-loops, WNK kinases also phosphorylate SPAK and OSR1 at their *C*-terminal domains.[4] Among the prominent *C*-terminal phosphorylation sites on SPAK and OSR1 is the WNK-phosphorylation of a highly conserved serine residue (S373 for SPAK and S325 for OSR1) (**Figure 1A** and **B**). Although previous studies showed that, unlike *T*-loop phosphorylation (A, **Figure 1A**), WNK-phosphorylation at S373 for SPAK and S325 for OSR1 does not affect their catalytic activities,[2, 4] the role of these phosphorylation sites remained unknown. Herein, we show that OSR1 binds the Cullin RING-E3 ubiquitin ligase complex Cul4-DDB1-WDR3/WDR6 (CRL4) and its phosphorylation at S325 by WNK kinases under osmotic stress compromises such binding.

Given our previous success with using biotinylated peptides and pulldown assays in identifying proteins that bind SPAK and OSR1 kinases in a phosphorylation-dependent manner,[8] our pursuit of understanding the function of OSR1 S325 phosphorylation by WNK kinases started by synthesizing two *N*-terminally

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biotinylated 19-mer peptides, which correspond to amino acids 316-334 (KKVRRVPGS**S**GRLHKTEDG) of human OSR1, with one of them having S325 phosphorylated and the other unphosphorylated (**Figure 2A**). These peptides were subsequently incubated with HEK293 cell lysates and then underwent biotin-streptavidin pull down. As controls, we used an *N*-terminally biotinylated 18-mer RFQV (SEEGKPQLVGRFQVTSSK), which is known to bind endogenous OSR1 and SPAK, and its corresponding single point mutant AFQV peptide (SEEGKPQLVGAFQVTSSK), which does not bind SPAK and OSR1.[2] The pulled down material underwent mass spectrometry analysis to identify the proteins that bound to these peptides (**Figure 2B**).

The results showed that the unphosphorylated OSR1 S325 peptide pulled down the E3 RING ubiquitin ligases Cul4A and Cul4B as well as their known protein binder DNA damage-binding protein 1 (DDB1)[9] (**Figure 2B**). Intriguingly, the peptide with a phosphoserine corresponding to WNK-phosphorylation at OSR1 S325 (and SPAK S373) did not show binding to Cul4 or DDB1. This suggested that Cul4 and DDB1 binding to OSR1 and SPAK kinases is affected by their WNK-phosphorylation at S325 and S373, respectively. As well as binding to DDB1, Cul4 is established to bind its substrates via DDB1-Cul4-associated factors (DCAF) proteins.[10] In our mass spectrometry data, two DCAF proteins, namely WD repeat-containing proteins (WDR) 3 and 6, were identified (**Figure 2B**).

In order to further confirm the binding, we subsequently repeated the pull-down assay from HEK293 cell lysates using the aforementioned peptides and employed Western blotting for the indicated proteins (**Figure 2C**). The results provided further evidence that the unphosphorylated OSR1-derived peptides bound to endogenous Cul4B and DDB1, while the binding to Cul4A was rather weak by Western blotting. Additionally, there was also binding observed to endogenous WDR3 and WDR6 though the commercially available antibodies for these proteins were not very sensitive or clean. Notably, as observed in the mass spectrometry data (**Figure 2B**), the binding of these proteins to OSR1 occurred with the non-phosphorylated OSR1 S325 and not the phosphorylated one (**Figure 2C**).

The most studied DCAF protein of CRL4 E3 Ubiquitin ligase is cereblon,[11] which binds the agent thalidomide and its analogues leading to the activation of Cul4 to degrade substrate proteins such as Aiolos.[12] To check if thalidomide and its analogues could lead to the degradation of SPAK and OSR1 kinases, we employed HEK293 cells, which express the CRL4 complex and Aiolos proteins. Treatment of HEK293 cells with thalidomide led to the degradation of Aiolos proteins as expected whereas no changes of SPAK and OSR1 total protein levels were observed (**Supporting Figure S1**). This suggested that cereblon is not the DCAF protein that mediates the binding of OSR1 and SPAK kinases to the CRL4 complex, but rather WDR3 and WDR6 proteins that were detected by the mass spectrometry and Western blotting (**Figures 2B** and **C**).

Next, we studied whether the observed CRL4 complex binding to the OSR1-derived peptide could also occur with endogenous and overexpressed SPAK and OSR1. First, we overexpressed in HEK293 cells GST-tagged OSR1 with either Myc-tagged Cul4A or B, left the cell unstimulated or underwent treatment with hypotonic buffer to active WNK kinases that phosphorylate OSR1 at S325 and SPAK at S373.[3] Following GST-pull down, the

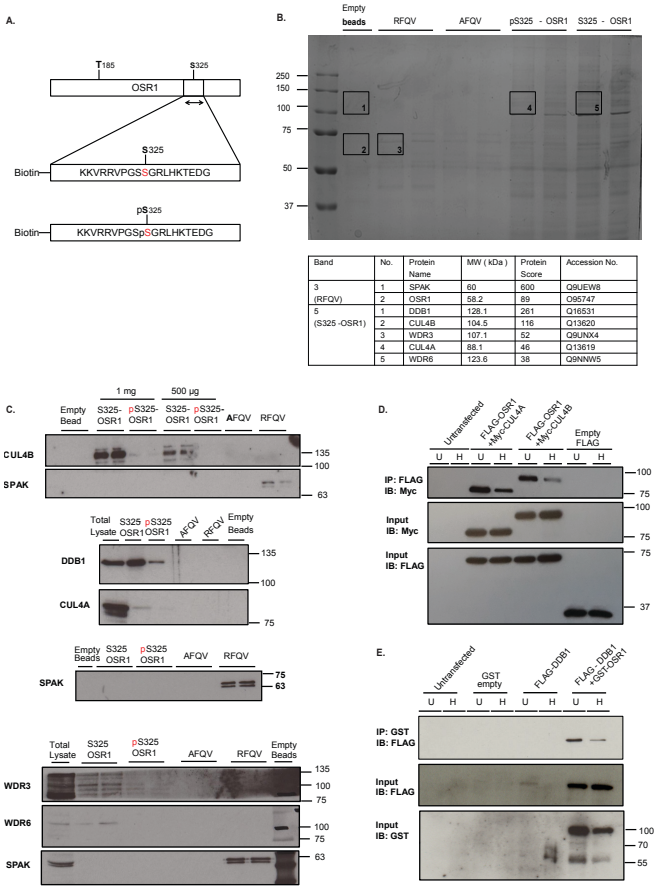

Figure 2. The CRL4 complex binds OSR1. **A.** Peptide sequences of the phosphorylated (pS325) or non-phosphorylated (S325) 19-mer OSR1 peptides (KKVRRVPGS**S**GRLHKTEDG) that were used in the pull down experiments. The peptides carry *N*-terminally biotin. **B.** Comassie stain of an SDS-PAGE gel showing the bands observed following the gel electropherosis of the material that was pulled down using the various peptides. The RFQV (SEEGKPQLVGRFQVTSSK) peptide, which binds SPAK and OSR1 , and its derivative AFQV (SEEGKPQLVGAFQVTSSK) peptide, which does not bind SPAK and OSR1, are derived from the human WNK4 kinase. They are also *N*-terminaly biotinylated and they were used in this experiment as controls. The bands in squares correspond to the bands that showed noticable difference between pS325 and S325 peptides. All of the indicated bands were excised and analysed by mass spectrometry. The table lists the proteins that comprise the Cullin-RING ligase with Cul4 (CRL4) and had a Mascot Score ≥ 30. **C.** Western blotting verification of the binding of Cul4A/B, DDB1, and WDR3/6 to the phosphorylated and non-phosphorylated peptides following straptavidine-biotin pulldown from HEK293 cell lysates. The RFQV and AFQV peptides were used as controls. **D.** Western blot verification of the binding of overexpressed full length FLAG-OSR1 to Myc-tagged full length Cul4A and B under untreated (U) or hypotonic (H) conditions [30 min treatment] after FLAG-pull down from HEK293 cells and immunoblotting for Myc. **E.** Western blot showing the binding of overexpressed GST-OSR1 full length to FLAG-DDB1 following GST-affinity pull down from HEK293 cells and immunobloting for FLAG. Prior to the pull down, the cells were left unstimulated (U) or were treated with hypotonic buffer (H) for 30 min.

material underwent Western blotting for Myc and the results showed that both Myc-tagged Cul4A and B bound the full length GST-OSR1 though the binding was observed most under the untreated condition and to a lesser extent in cells where OSR1 was phosphorylated at S325 because the treatment with the hypotonic buffer to activate WNK kinases (**Figure 2D**). Also, the overexpression of full-length GST-OSR1 with FLAG-tagged DDB1 in HEK293 cells that were either left unstimulated or treated with hypotonic buffer followed by pull down indicated that DDB1 binds OSR1 when it is unphosphorylated at S325 (untreated), but not when phosphorylated (hypotonic) (**Figure 2E**). These observations are in line with the OSR1 S325-phosphorylation

effect on its binding to Cul4A/B and DDB1 seen with the mass spectrometry and Western blotting data (**Figure 2B** and **C**, respectively).

For binding at the endogenous level, SPAK and OSR1 kinases were immunoprecipitated from HEK293 cells, that were left untreated or treated with hypotonic buffer to activate WNK-signaling. The immunoprecipitated material subsequently underwent Western blotting for DDB1 and WDR6 (**Supporting Figure S2**). The results showed that these proteins bound SPAK and OSR1 at the endogenous level further supporting the findings shown in **Figures 2B**-**E**. Attempts at verifying SPAK and OSR1 kinases binding to the Cul4A/B, and WDR3 at the endogenous level were limited by poor commercially available antibodies for these proteins.

To provide evidence the region where the CRL4 components bind OSR1, we conducted another pull down assay from HEK293 cell lysate from cells that had been co-transfected with the CRL4 components and GST-OSR1 full length and its GST-tagged fragments (Figure 3A). These fragments were the kinase domain without the *S*-motif (1-311), kinase domain plus the *S*-motif (1-363), OSR1 fragment without the *C*-terminal domain (1-435) and the C-terminal domain only (429-end) (**Figure 3A**). Following co-transfection, the cells were left unstimulated or treated with hypotonic buffer. Upon cell lysis, the total cell lysates underwent Western blotting to check the protein expression (**Supporting Figure S3**). The cell lysates were then used in affinity pull down experiments followed by Western blotting. As shown in **Figure 3**,

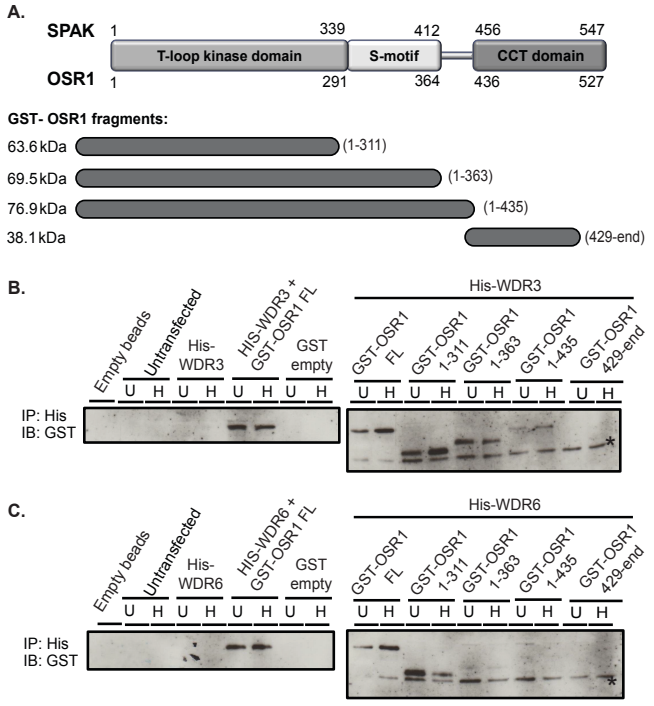

Figure 3. The CRL4 complex binds to the kinase domain of OSR1. A. Schematic diagram of the GST-OSR1 fragments that were used in pull down assays. **B**. GST-OSR1 and its fragments were co-expressed with His-WDR3 in HEK293 cells. Total protein lysate was prepared three days post-transfection and immunoblotting analysis was conducted after His pull down. Anti-GST antibody was used to detect the binding of OSR1 full length and its fragments to His-WDR3. **C**. As in C, using His-WDR6 and anti-GST antibodies. * indicates non-specific bands. In all cases, the cells were left unstimulated (U) or stimulated with hypotonic buffer (H) for 30 min.

the full length human OSR1 bound to full length WDR3 and 6, which further confirms the previous results shown in **Figure 2**.

Additionally, the binding of OSR1 to these proteins was more prominent in the untreated conditions than in the samples that had been treated with hypotonic buffer to phosphorylate the *S*-motif of the OSR1 by WNK kinases. In terms of the OSR1 fragments, the OSR1 kinase domain (1-311) showed binding to WDR3 and 6 that was more pronounced in the untreated samples as compared to the hypotonic ones. This was rather intriguing because this OSR1 kinase fragment does not contain part of the OSR1 *S*-motif, yet binding to WDR3 and 6 was achieved (**Figure 3B**-**C**). This suggests that OSR1 binding to WDR3 and 6 proceeds via the kinase domain, but is further mediated by the *S*-motif. Given the large size of these proteins (> 100 kD), such notion may be possible. In terms of the other OSR1 fragments, WDR3 showed more consistent binding to OSR1 fragments that contained the *S*-motif with S325 as expected (**Figure 3B**), whereas WDR6 binding to OSR1 1-363 and 1-435 was much weaker (**Figure 3C**). Notably, the OSR1 *C*-terminal domain (429-end), which lacks the kinase domain and *S*-motif, did not show any binding to WDR3 or 6 (**Figure 3B**-**C**). In general, the results shown in **Figure 3** support the earlier finding that WDR3 and 6 bind OSR1 through the *S*-motif and the kinase domain.

To get an insight into the function of the interaction between OSR1 and CRL4, we examined whether CRL4 ubiquitylates and degrades OSR1. For this, we co-expressed FLAG-OSR1 and HA-tagged ubiquitin (HA-Ub) in HEK293 cells and treated the cells with a proteasome inhibitor MG132[13] and Nedd8 activator enzyme inhibitor MLN4924[14] under basal and hypotonic condition. Prior to the pull-down assays, the total cell lysate from each condition underwent Western blotting to confirm the expression levels of FLAG-OSR1 and HA-ubiquitin (**Figure 4**). Additionally, an immunoblot of Cullin 2 (Cul2) was also carried out as a read out of MLN4924 ability to inhibit neddylation. The results showed that in the samples treated with MLN4924, which is an inhibitor of RING E3 ubiquitin ligases, Cul2 appeared as a single band since the smaller upper band of Cul2, which corresponds to neddylated Cul2 (Cul2-Nedd), disappeared (**Figure 4**). This confirms the inhibition of neddylation in the samples that were treated with MLN4924. Furthermore, a Western blotting of the total cell lysate of each sample with HA antibody gave a significant signal in the samples treated with the MG-132 proteasome inhibitor. This inhibition was comparable in the untreated and hypotonic samples as expected since MG-132 inhibits the proteasomal degradation of all of the cell's proteins.

Subsequently, FLAG-tagged OSR1 was pulled down from each sample and the pulled down material was probed with an anti-HA antibody by Western blotting. The results gave strong HA-ubiquitin signals only in the samples treated with the MG-132 proteasomal degradation inhibitor as expected (**Figure 4**). Intriguingly, the data showed that OSR1 ubiquitylation was more pronounced in basal (untreated) condition compared to the hypotonic condition (**Figure 4**). This implied that ubiquitylation of OSR1 is reduced by the stimulation of WNK-signaling (hypotonic), which leads to its *S*-motif phosphorylation. This observation is in line with the earlier findings that the CRL4 complex binds OSR1 under basal conditions and not under osmotic stress due OSR1 phosphorylation on the *S*-motif.

The fact that the Cul4-DDB1-WDR3/6 complex binds to SPAK and OSR1 kinases in resting conditions prior to osmotic stress-induced phosphorylation by WNK kinases suggests that this ubiquitin ligase system may constitutively ubiquitylate SPAK and

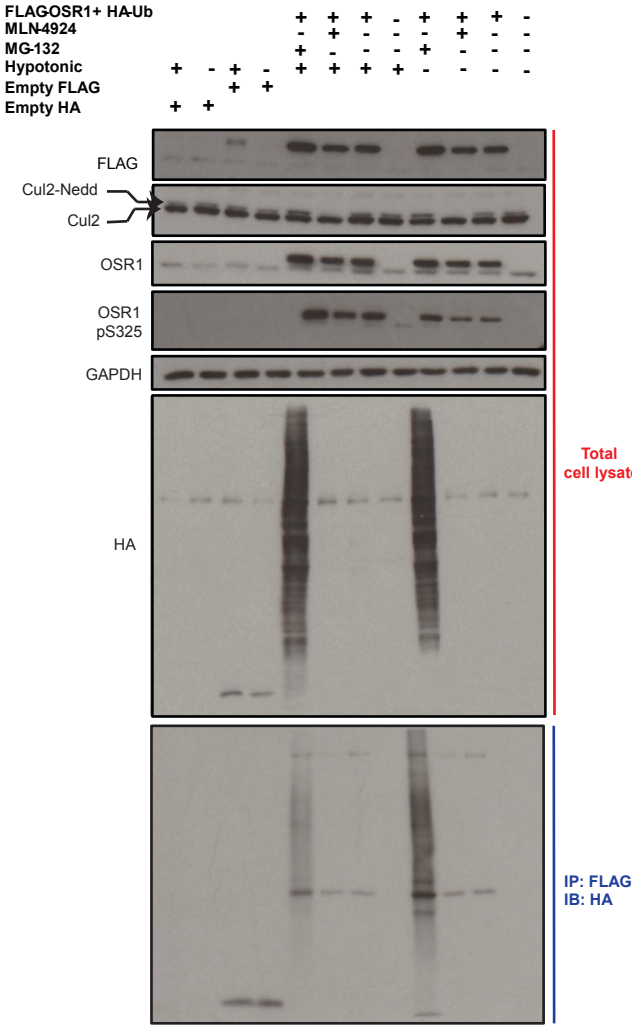

Figure 4. OSR1 ubiquitylation is inhibited under osmotic stress. FLAG-OSR1 and HA-ubiquitin (HA-Ub) were co-expressed in HEK293 cells. The cells were left untreated or treated with MLN4924 (1 μM for 6 h) or MG-132 (10 μM for 24 h). Prior to cell lysis, the cells were either left unstimulated or were treated with hypotonic buffer. Upon lysis, the cell lysates underwent Western blotting for total FLAG, Cul2, OSR1, GAPDH and Ub. Also, FLAG-pull down was carried out and pulled down material was probed with anti-HA antibody by Westen blotting.

OSR1 kinases under resting conditions, a physiological phenomenon that has been observed with other proteins.[15] Such ubiquitylation is then inhibited under osmotic stress as a result of SPAK and OSR1 *S*-motif phosphorylation due to the loss of the Cul4-DDB1-WDR3/6 complex binding to SPAK and OSR1 kinases following S-motif phosphorylation as indicated by the data presented herein. The type and function of such constitutive ubiquitylation needs to be further investigated. Notably, beyond the loss of the CRL4 complex binding to SPAK and OSR1, the S-motif phosphorylation at other serine residues by WNK kinases, namely S387 for SPAK and S339 for OSR1 is known to mediate the binding of SPAK and OSR1 kinases to the scaffolding protein MO25.[8] This is now understood to be the second part of SPAK and OSR1 kinases activation as initially osmotic stress leads to SPAK and OSR1 phosphorylation on their T-loop by WNK kinases[4] and this is subsequently followed by binding to MO25, which leads to 80- to 100-fold increase in their kinase catalytic activity.[6]

In conclusion, this work provides the first example of an E3 ubiquitin ligase system that binds SPAK and OSR1 kinases. Critically, it provides a new molecular insight that links the CRL4 complex to ion homeostasis and the regulation of blood pressure through SPAK and OSR1 kinases. Such notion is supported in some studies where deletion of Cul4A resulted in changed in blood pressure.[16] This novel finding provides a new avenue for understanding the fundamental biology of ion homeostasis and the biology of diseases that involve salt imbalances and those where the CRL4 components are mutated. Critically, it highlights a potential new role for small molecule CRL4 binders as modulators of ion homeostasis.

Experimental Section

Full experimental details are provided in the Supporting Information.

Acknowledgements

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Keywords: SPAK • OSR1 • Cullin • Phosphorylation • Ubiquitylation

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Supporting Information

I. MATERIALS

A. Reagents. The peptides used in this work were purchased from GL Biochem (China) and their sequences were: RFQV (Biotin-C_6-SEEGKPQLVGRFQVTSSK), AFQV (Biotin-C6- SEEGKPQLVGAFQVTSSK), S325-OSR1 (Biotin-C_6-KKVRRVPGSSGRLHKTEDG), pS325-OSR1 (Biotin-C_6-KKVRRVPGSpSGRLHKTEDG). Thalidomide (T144), DMEM (D6429), LB broth (L7275), LB agar (L7025) and ampicillin 100mg/mL (A5354), Fetal Bovine Serum (F0804) and PenStrep (Gibco™ Penicillin-Streptomycin 10,000 (U/mL) were purchased from Sigma-Aldrich (UK).

B. Antibodies. Anti-OSR1 (S636B), anti-SPAK (S551D and S637B) and anti-SPAK pS373 (S670B) were purchased from the MRC PPU Reagents and Services, University of Dundee (UK). Anti-GAPDH rabbit mAb (#2118), anti-SPAK (#2281), anti-CUL4A (#2699), anti-DDB1 (#5428), anti-HA (#3724), anti-Aiolos (#15103), anti-Ubiquitin (#3933), anti-Myc HRP-conjugated (#14038), anti-DYKDDDDK (#14793), anti-rabbit IgG HRP-linked antibody (#7074), anti-mouse IgG HRP-linked antibody (#7076) were purchased from Cell Signalling Technology. Anti-CUL2 (ab166917), Anti-DDDDK (ab45766) and rabbit anti-Sheep IgG H&L HRP (ab97130) were purchased from Abcam. Anti-CUL4B (C9995) was purchased from Sigma-Aldrich (UK). Anti-GST (8-326) was purchased from Thermo Scientific, anti-WDR3 (102-1161) was purchased from RayBiotech, and anti-WDR6 (PA5-41984) was purchased from Invitrogen.

C. Plasmids. Plasmid pEBG-GST-HA-OSR1 (DU44767), pCMV5-HA-Ubiquitin (DU3650), pCMV5-FLAG-OSR1 (DU6194) and pEBG-GST empty were purchased from the MRC PPU Reagents and Services, University of Dundee (UK). pEBG-GST-HA-OSR1 containing GST-HA-OSR1 insert in pEBG6P as parent plasmid. pCMV5-HA-Ubiquitin expressing HA-Ub from pCMV5 as parent plasmid which was inserted by HA-Ub in *BamH*I restriction site as *N*-terminal insert for mammalian expression. pCMV5-FLAG-OSR1 containing FLAG-OSR1 was inserted in parental plasmid pCMV5-FLAG1. The plasmids of pcDNA3-Myc3-Cul4A (#19951), pcDNA3-Myc3-Cul4B (#19922), pcDNA3-FLAG-DDB1 (#19918), and pcDNA5-FRT/TO-Venus-Flag (#40998) as FLAG empty vector were purchased from Addgene. The gene of interests in all the plasmids were inserted in *N*-terminal of the parental plasmid. Myc3-Cul4A was inserted in pcDNA3 *Eco*RI (5') and *Apa*I (3') cloning site. pcDNA3-Myc3-CUL4B with CMV as promoter was inserted in pcDNA3-Myc3 as vector backbone. The FLAG-DDB1 insert in pcDNA3 as parent plasmid also used CMV as promoter. The plasmids of pcDNA5D-FRT-TO-6xHis-WDR3 (DU63480) and pcDNA5D-FRT-TO-6xHis-WDR6 (DU63606) were purchased from the MRC PPU Reagents and Services, University of Dundee (UK). Both plasmids were inserted in *Bam*HI restriction site for mammalian expression. The plasmids for the fragments of GST-HA-OSR1 (pEBG6P GST-HA-OSR1 1-311 (DU6018), pEBG6P-GST-HA-OSR1 1-363 (DU6019), pEBG6P-GST-HA-OSR1 1-435 (DU6020) and pEBG6P-GST-HA OSR1 429-end (DU6586) were also obtained from the MRC PPU Reagents and Services, University of Dundee (UK). The GST-HA was inserted as *N*-terminal of OSR1 in *Bam*HI restriction site of pEBG6P parental plasmids for mammalian expression.

II. METHODS

A. Cell culture. HEK293 cells were cultured in DMEM (D6429, Sigma) supplemented with 10% Fetal Bovine Serum (FBS) and 1% of PenStrep. HEK293 cells were maintained in T75 flasks at 37°C and 5% CO_2. Subculturing was done when the cells reach 80-90% confluency. Hypotonic condition was applied to stimulate WNK signalling by replacing media with hypotonic low Cl^- buffer pH 7.4 (67.5 mM sodium gluconate, 2.5 mM potassium gluconate, 0.5 mM $CaCl_2$/$MgCl_2$, 1mM Na_2HPO_4/Na_2SO_4 and 7.5 mM HEPES) [1] for 30 minutes incubation at 37 °C, and under 5% CO_2.

B. Preparation of total protein lysate and protein concentration measurement. The cells were lysed when they were at 70-80% confluency. The cells were first washed with Phosphate Buffer Saline (PBS) (D8662, Sigma) and then 300-400 µL lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.3% CHAPS, 0.27 M sucrose, 1 mM Na_3VO_4, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM benzamidine and 0.1 mM PMSF) was used to each 10 cm dish. The cells were scrapped, and the lysates were transferred to microtubes and spun down at 10,000 rpm for 10 minutes at 4 °C. Finally, the supernatant was transferred to new microtubes and stored at -20°C. Protein concentration was measured using Bradford Assay[2]. 0.125; 0.25; 0.5 and 1 mg/ml of Bovine Serum Albumin (BSA) was used as protein standard in the Bradford assay.

C. Peptide Pull Down. Streptavidin sepharose high performance beads (GE Healthcare), pre-washed with buffer A (10 mM Tris-HCl (pH 8), 0.1 mM EGTA), were incubated with 1 mg of HEK293 total protein lysate at 4 °C for 10 min and then washed with buffer A as a pre-clearing step. This was carried out three times. Upon the third wash with buffer A, the pre-cleared supernatant was incubated on ice with 3 µg of the relevant peptide for 10 min. 20 µL streptavidin beads, which had been pre-washed with buffer A, were then added to the

HEK293 protein lysate/peptide mixture and incubated for 5 min at 4 °C in rolling shaker. Finally, the beads were washed twice with CHAPS lysis buffer and twice with buffer A.

D. Immunoprecipitation. Anti-SPAK or anti-OSR1 antibodies were conjugated to protein G-sepharose beads (PC-G5, Generon) via the covalent cross-linking method using dimethyl pimelimidate (D8388, Sigma-Aldrich) with ratio 1:1.[3] Prior to the immunoprecipitation, HEK293 total protein lysates were pre-cleared by incubation with protein G-sepharose beads at 4 °C for 10 min and washed with buffer A. This pre-clearing step was carried out three times. 1 mg of pre-cleared lysates were then incubated overnight in rolling shaker at 4 °C with 20 µL of anti-SPAK or anti-OSR1 antibody-protein G-sepharose bead conjugates. Next, the beads were washed twice with CHAPS lysis buffer and twice with buffer A. The collected beads were then prepared for SDS PAGE, at a final concentration of 1 µg/ µL, by adding 1x SDS loading buffer and boiling 95 °C for 5 minutes.

E. GST Pull Down. 40 µL of GST beads (Glutathione Sepharose High Performance, GE Healthcare) were first washed with 1 mL PBS or buffer A three times. Once washed, the GST beads were incubated at 4 °C overnight with 1 mg HEK293 total protein lysates that overexpress the GST tagged protein of interest. The beads then were collected by centrifugation 500 rpm, 1 min at 4 °C. Subsequently, 1 mL of CHAPS lysis buffer and Buffer A were used to wash the beads (twice with each buffer). Finally, 100-150 µL of 1x SDS loading buffer was added to the beads, gently mixed and boiled at 95°C for 5 minutes.

F. FLAG Pull Down. The resin beads (Anti-FLAG M2 affinity gel, Sigma Aldrich UK) were washed with TBS buffer (50 mM Tris-HCl, 150 mM NaCl pH7.4, 0.1 M glycine pH 3.5) three times. 40 µL of FLAG beads were then equilibrated with TBS buffer before being incubated 1 mg total protein lysate overnight at 4 °C. Following centrifugation, the FLAG beads were washed three times with TBS buffer and 1x SDS loading buffer was added. The suspension was then boiled for 95 °C for 5 and used in immunoblotting studies.

G. His Pull Down. His pull down was performed using HisPur Ni-NTA resin bead (Thermo Scientific). 1 mg of HEK293 total protein lysate was incubated with 500 µL of binding buffer (20 mM Tris-HCl pH8.0, 100 mM NaCl, 30 mM Imidazole and 10 µg/ml BSA) and 20 µL Ni-NTA resin bead at 4°C overnight. The beads were then pelleted by centrifugation, 700 rpm for 1 minute, and washed three times with the washing buffer (20 mM Tris-HCl pH8.0, 200 mM NaCl, 50 mM imidazole and 0.2% Tween-20). 100 µL of 1xSDS loading buffer was then added to the beads and the suspension was boiled (95 °C) and 5 minutes before being used in the immunoblotting studies.

H. Immunoblotting. 15-20 µg of total protein was loaded per well, and these were subjected to separation in polyacrylamide gels containing 1% SDS and transfer onto nitrocellulose membrane. The membranes were blocked in 10% skimmed milk in TBST (50 mM Tris/HCl (pH 7.5), 0.15 M NaCl containing 0.25% Tween-20) for 30 minutes at room temperature. The membranes were then incubated with the respective primary antibodies in 5% BSA or 5% skimmed milk in TBST overnight at 4 °C or 1 hour at room temperature. Following the washing of the membranes with TBST (five times, 5 minutes per wash), the membranes were then incubated with the respective secondary antibodies for 1 hour at room temperature. Following the final wash of the membranes with TBST (five times, 5 minutes per wash), protein detection on the membranes was performed using horseradish peroxidise-conjugated secondary antibodies and the ECL® reagent (Amersham Bioscience).

I. Protein overexpression and co-overexpression. Transformation of all cDNA plasmids to *E.coli* DH5α (Invitrogen) was done by heat shock at 42 °C for 90 seconds. The mixture was then spread on LB agar plate supplemented with 100 µg/µL ampicillin and incubated at 37 °C overnight. The grown colonies were grown in 500 mL LB media supplemented with ampicillin (100 µg/µL) at 37 °C overnight with 180 rpm shaking to increase the copy number of recombinant DNA. Plasmid DNA extraction from the overnight cultures was performed using Qiagen Plasmid Maxi Kit (Cat. No.12163) following the company's kit protocols. The DNA concentration was determined using PHERAstar FS (BMG Labtech) and NanoVue Plus (GE Healthcare Lifesciences) was used to determine the quality of the isolated DNA. Finally, cDNA plasmids were transiently transfected or co-transfected in HEK293 cells using polyethylenimine (PEI) (Sigma-Aldrich) in serum and free antibiotic growth media. Transfected cells were then lysed 2-4 days post-transfection. The lysates from each experiment underwent pulldown experiments as described above.

J. Mass Spectrophotometry analysis for protein identification. After protein separation by SDS-PAGE and gel staining using NOVEX Colloidal Blue Staining Kit (LC6025, Invitrogen), the gel bands of interest were excised. The gel slices were then washed and lysed with trypsin before mass spectrophotometry (MS) analysis using previously reported protocols.[4]

K. MLN4924 and MG132 treatment on HEK293 cell. HEK293 cells were ca. 80% confluent when they were treated with MLN4924 (Biovision) and MG132 (LKT Laboratories). These compounds were initially prepared in 100 mM stock solutions in DMSO. The compounds were then further diluted down, in DMSO, and used to

treat the cells with MLN4924 (1 μM for 6 h), MG-132 (10 μM for 24 h) and a final concentration of DMSO not exceeding 0.1%.

L. HEK293 cells treatment with thalidomide. The cells were cultured in 6-well plates before treatment with the compounds. Thalidomide was prepared in 100 mM stock solution in DMSO. To achieve the final concentrations of the compound, serial dilution of stock solution was prepared so that the highest concentration of DMSO in the cells was 0.1%.

III. SUPPORTING FIGURES

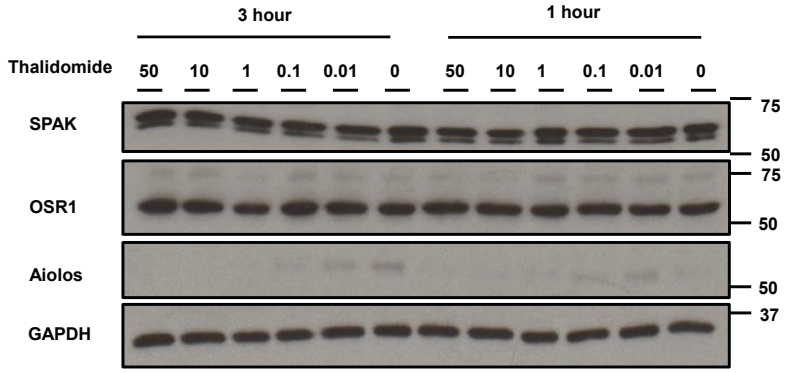

Figure S1. **Cereblon (CRBN) is not the substrate adaptor of CRL4 complex which binds to OSR1**. HEK293 cells were treated with thalidomide at the indicated concentrations and incubation times. Following lysis, the cell lysates underwent Western blotting for total endogenous SPAK and OSR1 kinases as well as the CRL4 substrate Aiolos. 20 ug of total protein was loaded in each sample. GAPDH was used as a loading control. The total levels of SPAK and OSR1 remained the same under all the thalidomide conditions while those of Aiolos, as expected, were reduced in a dose-dependent manner.

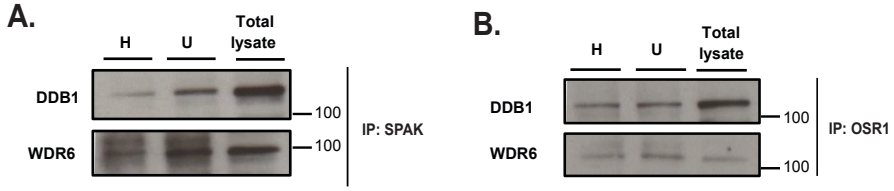

Figure S2. A Western blot showing the binding of various endogenous CRL4 complex proteins to endogenous SPAK and OSR1 following their immunoprecipitation from HEK293 cells. **A.** HEK293 cells were either left unstimulated (U) or treated with hypotonic low-chloride buffer (H) and then lysed. 3 mg of total protein cell lysate was incubated with anti-SPAK conjugated beads overnight to immunoprecipitate endogenous SPAK. Following the washing of the beads, the material left on the beads was treated with SDS sample buffer, and underwent Western blotting for DDB1 and WDR6 antibodies. **B.** As in **A.**, but immunoprecipitation was performed using Anti-OSR1 conjugated beads.

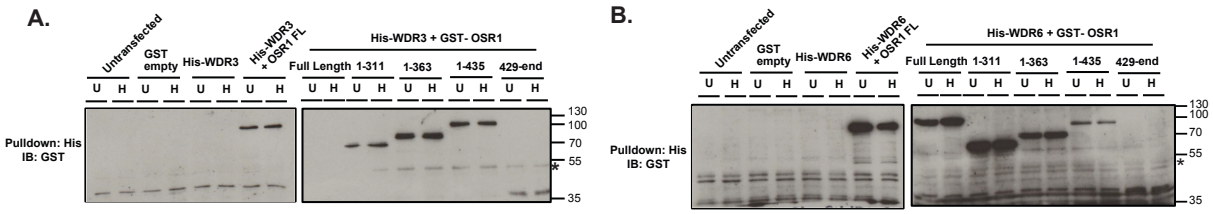

Figure S3. A Western blot showing the overexpression in HEK293 of GST-OSR1 full-length (FL) or its fragments with **A.** His-WDR3 and **B.** His-WDR6. Following the overexpression of the indicated proteins in HEK293 cells, the cells were lysed and immunoblotting for GST was performed and shown in this figure.

References

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