SUPPLEMENTARY-

Functional kinomics establishes a critical node of volume-sensitive cation-Cl^- cotransporter regulation in the mammalian brain

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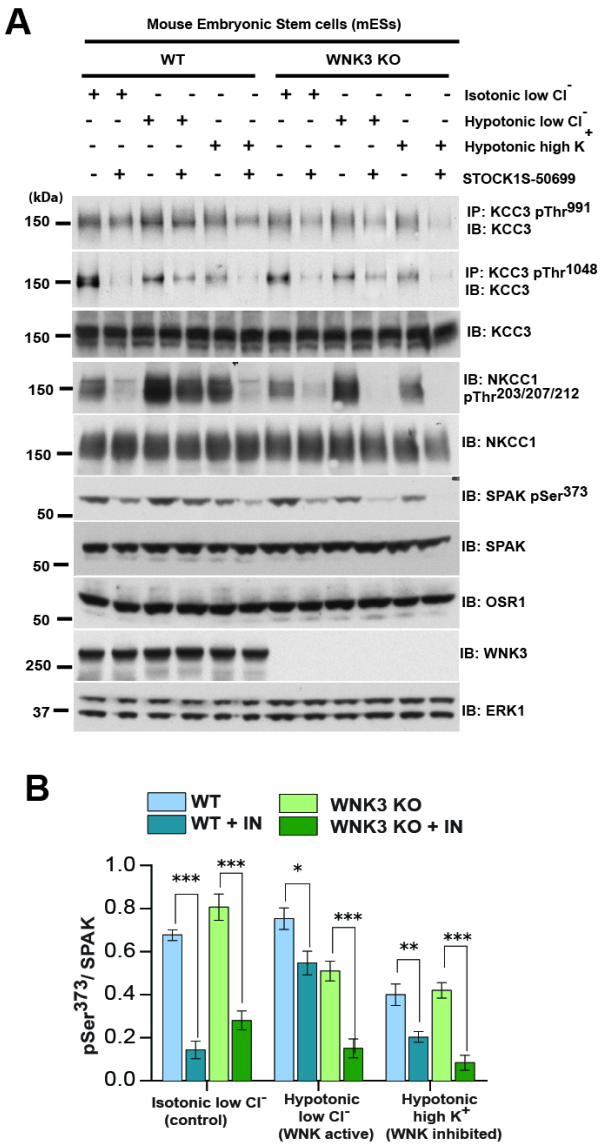
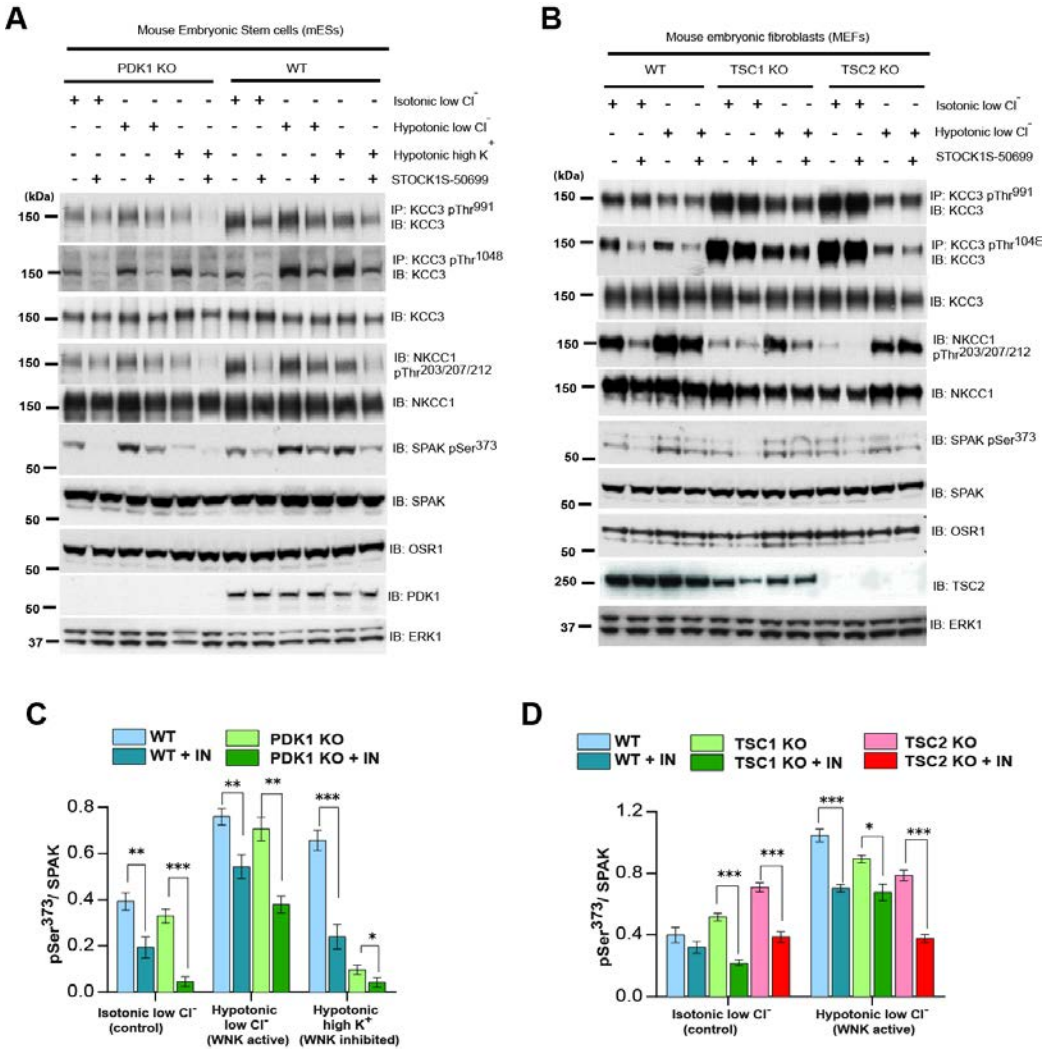
SUPPLEMENTARY FIGURES



Supplementary Figure 1: Validation of siRNA screen hits using knockout cell models.

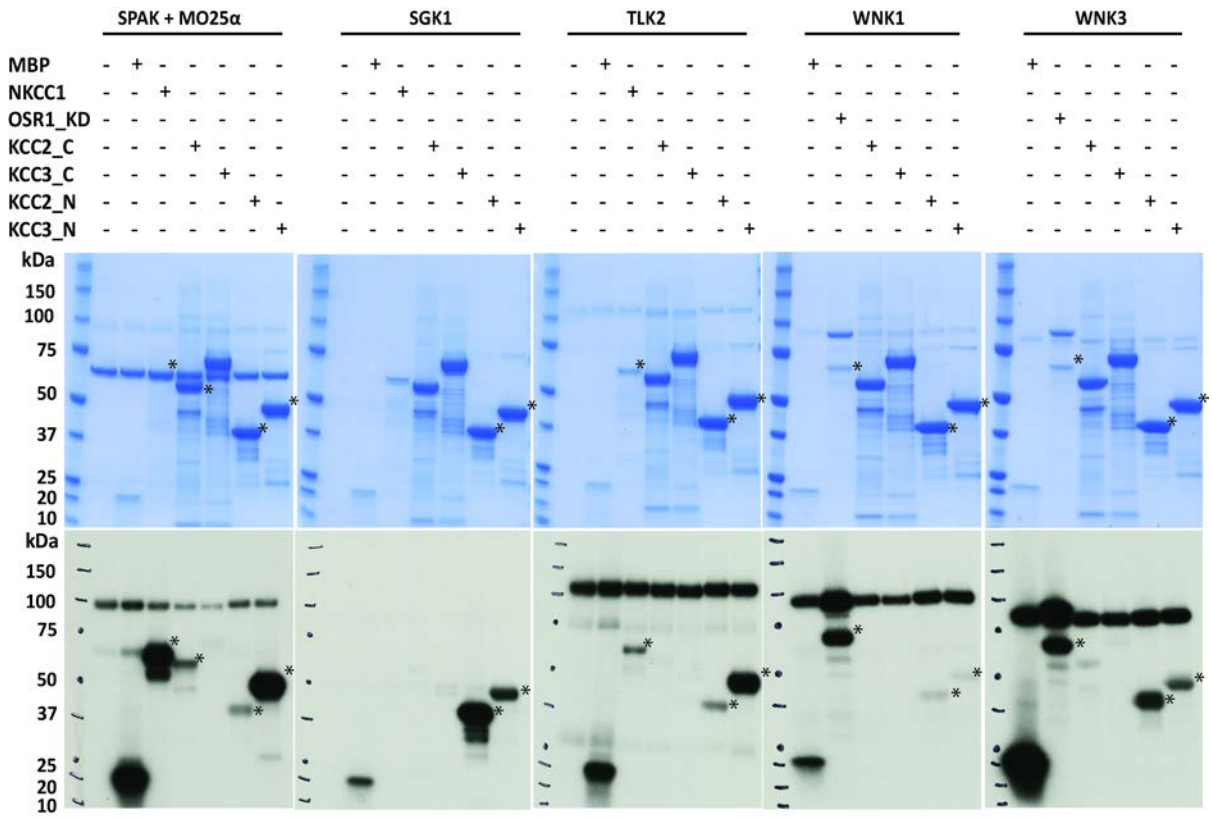
(A) WNK3-SPAK/OSR1 regulation and phosphorylation of endogenous KCC3. Matched WNK3 WT and WNK3 KO ES cells [1] were incubated 30 min with either control isotonic media, hypotonic low Cl^- media or hypotonic high K^+ media in the absence or presence of STOCKS1-50699 (IN). The lysates were then subjected to immunoblot with the indicated antibodies. Molecular masses (kDa) are at left. Similar results were obtained in 3 separate experiments. See **Figure 2A** for Western blot quantitation. **(B)** All analyses presented in **A** were also conducted in hypotonic high K^+ media which, like hypotonic low Cl^- media,

activates KCC3 and promotes its dephosphorylation. Results presented here are analogous to those for hypotonic, high K^+ media in **Figure 2A**.



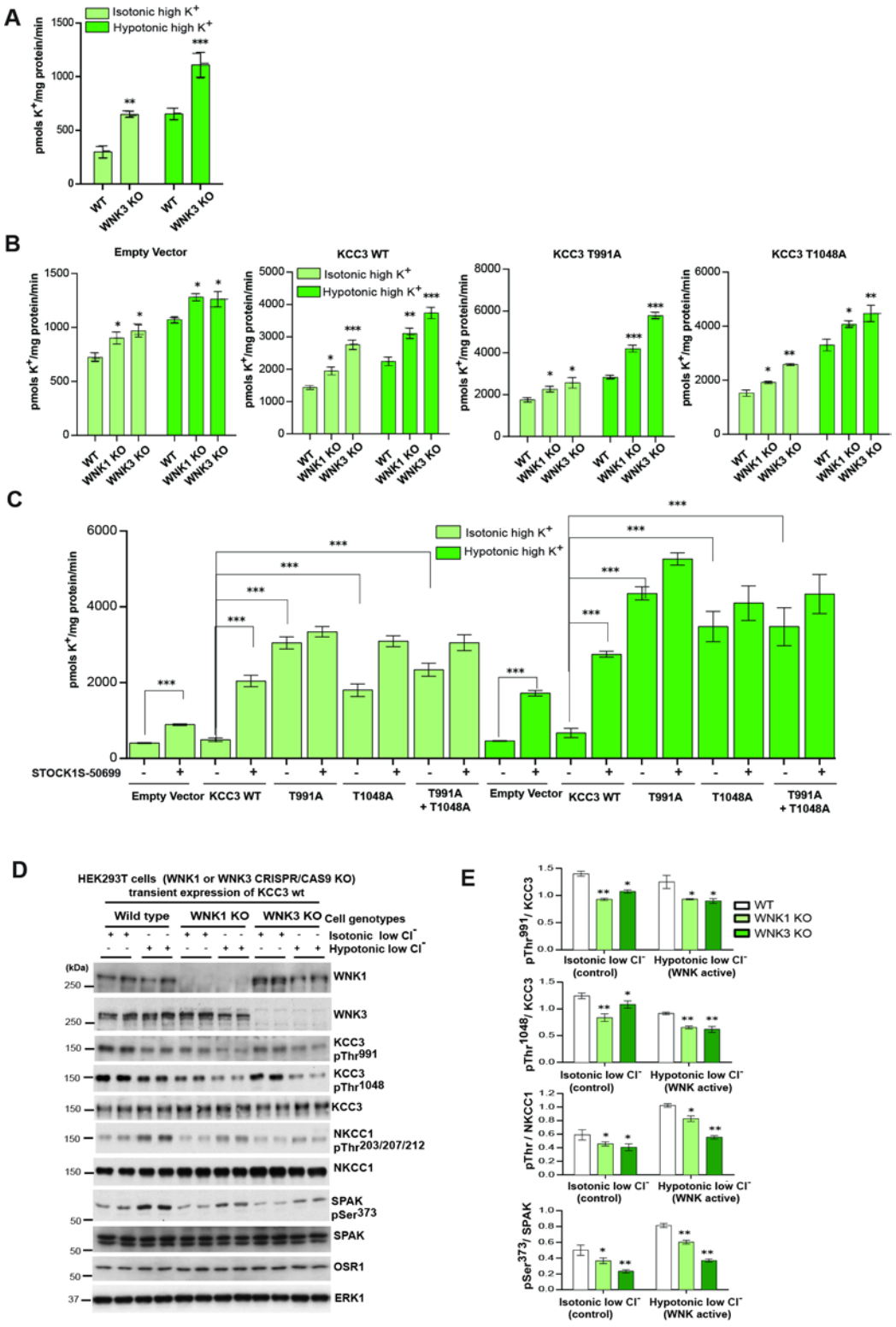
Supplementary Figure 2: Validation of siRNA screen hits using knockout cell models.

(A) PDK1 regulation and phosphorylation of endogenous KCC3. Matched WT and double PDK1 knock-out ES cells[2] were treated as in (Supplementary Figure 1A). See **Figure 2B** for Western blot quantitation. **(B)** TSC1 or TSC2 regulation and phosphorylation of endogenous KCC3. Previously described matched WT and TSC1 or TSC2 mouse embryonic fibroblast (MEF) cells[3], were treated as in (**Supplementary Figure 1A**). See **Figure 2C** for Western blot quantitation. **(C-D)** All analyses presented in **Supplementary Figure 2A and B** were also conducted in hypotonic, high K^+ media. Results presented here are analogous to those for hypotonic, high K^+ media in **Figure 2B and C**. IN, STOCK1S-50699.



Supplementary Figure 3: ***In vitro*** **kinase assays of candidate KCC3 regulatory kinases.**

WNK1 (residues 1-491), WNK3 (1-434), KCC2A-N (1-119), KCC2A-C (852-1039), KCC3A-N (1-175), KCC3A-C (886-1141), NKCC1 (1-288), OSR1 D164A (1-527) were expressed in bacteria. TLK2 (1-750) and SGK1 (60-431) were expressed in baculovirus. MBP (Sigma, M1891) and activated SPAK (Carna Biosciences, 07-130) were purchased. The N-terminal cytoplasmic domain and C-terminal cytoplasmic domain of KCC2A (5 µg) and KCC3A (5 µg) and control proteins MBP (0.5 µg), N-terminal cytoplasmic domain of NKCC1 (5 µg) and OSR1 D164A (5 µg) were incubated with 0.08 µM of SPAK (in the presence of 10-fold molar excess of MO25α), SGK1, TLK2, WNK1 or WNK3. Reactions were performed for 1 h at 30°C in a buffer containing 50 mM Tris-HCl (pH = 7.5), 0.1 mM EGTA, 5 mM $MgCl_2$, 0.1% Mercaptoethanol and 0.1 mM Mg-[γ^{32}P]ATP (~200 cpm/pmol) and stopped by adding LDS sample buffer. Phosphorylation of substrates was analysed following electrophoresis of the Colloidal blue-stained bands (upper panels) and autoradiography (lower panels). An asterisk indicates the bands corresponding to the NTD and CTD fragments. Molecular weight (kDa) is at left of each gel.



Supplementary Figure 4: WNK3-SPAK kinase regulates the phosphorylation and function of KCC3.

(A) $^{86}Rb^+$ uptake assays in WNK3 WT and KO ES cells. WT and WNK3 KO cells[1] were incubated with control isotonic high K^+ media or KCC3-activating hypotonic high K^+ media for 30 min in the presence of 1 mM ouabain and 0.1 mM bumetanide. $^{86}Rb^+$ uptake proceeded for 10 min and was then quantified by scintillation counting. ***, p<0.001; **, p<0.01; *, p<0.05, when compared to WT values under the same conditions. (**B**) $^{86}Rb^+$ uptake assays in WT, WNK3 KO and WNK1 KO HEK293T cells. The indicated cells were transfected with constructs encoding a Flag empty vector or the indicated WT or mutant constructs (against KCC3 Thr^{991} and Thr^{1048}) of N-terminal FLAG-tagged KCC3. 36 h post-transfection, cells were treated for 30 min with control isotonic high K^+ or hypotonic high K^+ conditions. 10 min $^{86}Rb^+$ uptake assays were then carried out in the presence of 1 mM ouabain and 0.1 mM bumetanide and quantitated by scintillation counting. Results are presented as means ± SEM for triplicate samples. ***, p<0.001; **, p<0.01; *, p<0.05, when compared to WT values under the same conditions. Cell lysates from in parallel experiment were also subjected to immunoblot analysis (**Supplementary Figure 4D-E**). (**C**) $^{86}Rb^+$ uptake assays in the absence and presence of STOCK1S-50699. HEK-293 cells were transfected and treated as in (B). 10 min $^{86}Rb^+$ uptake assays were carried out in the presence of 1 mM ouabain and 0.1 mM bumetanide plus/minus 10 μM STOCK1S-50699 and quantitated by scintillation counting of triplicate samples (Cmeans ± SEM). **(D)** as in (B) HEK293T WT, WNK3 KO and WNK1 KO cells (see Methods) were each transfected with WT KCC3, KCC3 Thr^{991}Ala and KCC3 Thr^{1048}Ala mutants, and were treated for the indicated times with hypotonic low Cl^- conditions or control isotonic low Cl^- conditions. Harvested cell lysates were subjected to SDS-PAGE and Western blotting with the indicated antibodies as described. Molecular masses (kDa) are indicated at left. **(E)** Graphs show quantitation of Western blot ratios (phospho-KCC3) / (total KCC3) (n=3, means ± SEM). ***, *p<0.001*; **, *p<0.01*; *, *p<0.05*; ns: non-significant (unpaired t-test). Under hypotonic low Cl^- conditions, WNK1 KO cells and WNK3 KO cells both exhibited decreased phosphorylation of heterologous KCC3 at Thr^{991} (*p<0.001*) and Thr^{1048} (*p<0.001*). WNK1 KO HEK293T cells and WNK3 KO HEK293T cells also exhibited apparent decreases in NKCC1 Thr^{203}/Thr^{207}/Thr^{212}, SPAK Ser^{373}, and OSR1 Ser^{325} phosphorylation (*p<0.05*).

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