SUPPLEMENTARY MATERIAL

Regulation of the p38-MAPK pathway by hyperosmolarity and by WNK kinases

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Running title: p38-MAPK regulation by hyperosmolarity and by WNKs

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SUPPLEMENTARY TABLES

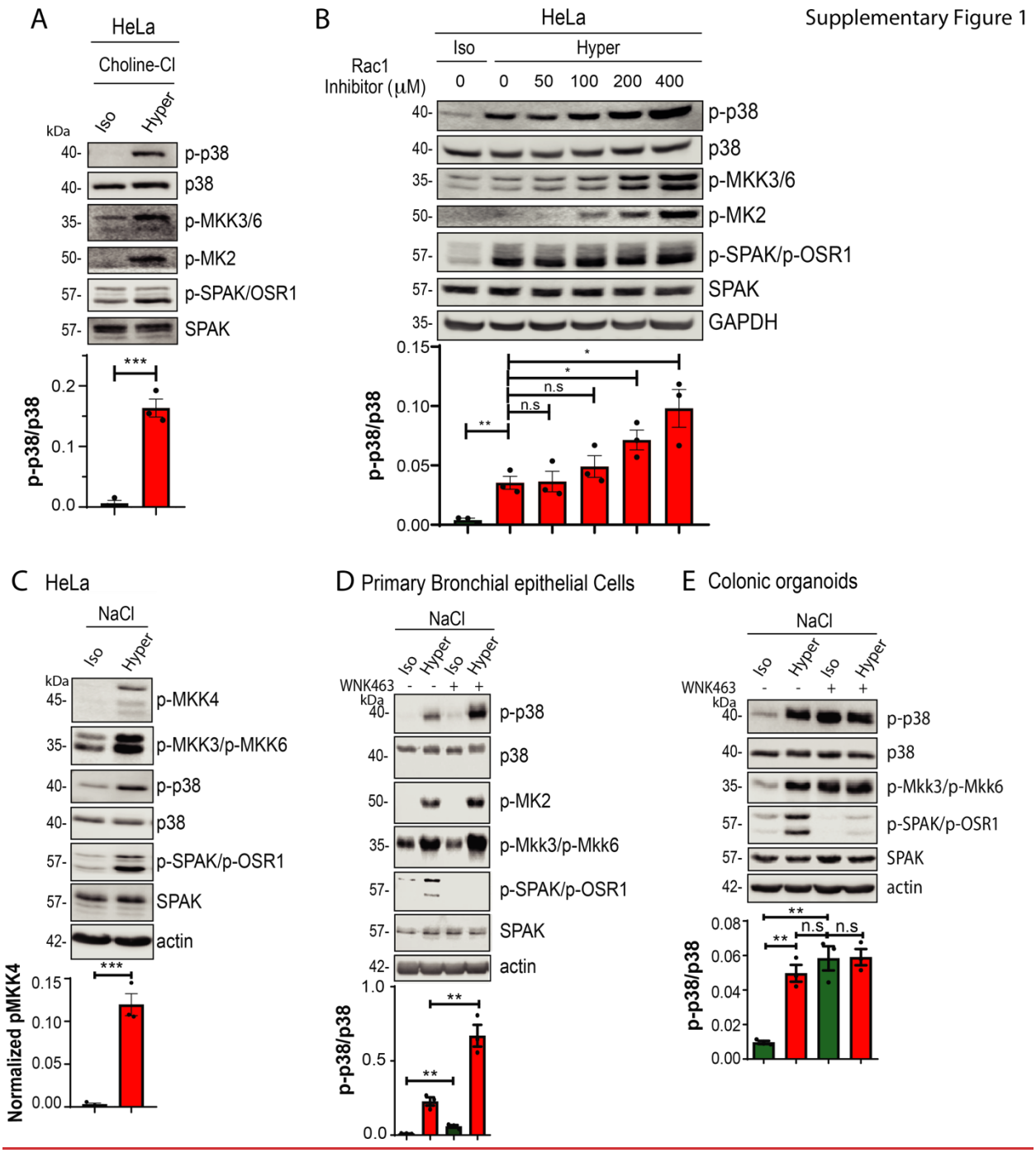
Table S1. Key resources of chemical, reagents and antibodies

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-SPAK (STK39)	Thermo-Fisher	Cat# MA5-24935
Mouse anti-β-actin	Sigma-Aldrich	Cat# A2228; RRID:AB_47669
Mouse anti-phospho-p38 MAPK (T180/Y182)	BD Bioscience	Cat# 612280; RRID:AB_399597
Mouse anti-vinculin	Santa Cruz	Cat# sc-25336; RRID:AB_628438
Rabbit anti-NKCC1	Cell Signaling	Cat# D13A9; RRID:AB_10830068
Rabbit anti-phospho-SPAK antibody (Ser-373)/phospho-OSR1 antibody (Ser-325)	EMD Millipore	Cat# 07-2273
Rabbit anti-phospho-MKK3 (Ser189)/MKK6 (Ser207)	Cell Signaling	Cat# 9231
Rabbit anti-phospho-SEK1/MKK4 (Ser257/Thr261)	Cell Signaling	Cat# 9156
Rabbit anti-phospho-ASK1 (Thr845) Antibody	Cell Signaling	Cat# 3765
Rabbit anti-p38 MAPK	Cell Signaling	Cat# 9212; AB_330713
Rabbit anti-GAPDH	Cell Signaling	Cat# 2118
Rabbit anti-phospho-MAPKAPK-2 (p-MK2) (Thr334)	Cell Signaling	Cat# 3007
Rabbit anti-WNK1	Cell Signaling	Cat# 4979
Rabbit anti-phospho-TRAF2 (Ser11)	Cell Signaling	Cat# 13908
Rabbit anti-TRAF2	Cell Signaling	Cat# 4724
Rabbit anti-phospho-TAK1 (Ser412)	Cell Signaling	Cat# 9339
Rabbit anti-TAK1	Cell Signaling	Cat# 5206
Rabbit anti-phospho-p70 S6 Kinase (Thr389)	Cell Signaling	Cat# 9205
Mouse anti-p70 S6 Kinase	Santa Cruz	Cat# SC-8418
Chemicals, Peptides, and Recombinant Proteins		
SB203580	SelleckChem	Cat# S1076
Monensin	Sigma-Aldrich	Cat# M5273
Wnk463	SelleckChem	Cat# S8358
5-(N-Ethyl-N-isopropyl) amiloride (EIPA)	Sigma-Aldrich	Cat# 1154-25-2
Gramicidin A	Sigma-Aldrich	Cat# 11029-61-1
Puromycin	Bioshop	Cat# PUR333
GS-444217	MedKoo Biosciences	Cat# 564706
5Z-7-Oxozeaenol	Tocris	Cat# 3604
NSC23766	Tocris	Cat# 2161
Cariporide	Tocris	Cat# 5358
Advanced DMEM/F-12	Thermo Fisher	Cat# 12634028
Intesticult Organoid Growth Medium (mouse)	Stem Cell	Cat# 06005
Corning Matrigel	Corning	Cat# 356231
Plasmids		
pcDNA3.1-MAP3K5 (ASK1) WT	Addgene	Plasmid#: 47104
pcDNA3 HA-humanTRAF2	Addgene	Plasmid#: 66932

pEBB-3XMyc-TRAF2	Addgene	Plasmid#: 44104
pEBB-3XMyc-TRAF2 S11A	Addgene	Plasmid#: 44105
Experimental Models: Cell Lines		
HeLa	ATCC	Cat#CCL-2
HEK293	ATCC	Cat#CRL-1573
MDCK	ATCC	Cat#CCL-34
Human Primary Bronchial Epithelial Cells	Abm	Cat# T4006
Scramble Control KD HeLa	(14)	
NKCC1 KD HeLa	(14)	
Recombinant DNA		
pGIPZ human TNFR1 shRNA	Dharmacon	V2LHS 94071
pGIPZ human WNK1 shRNA	Dharmacon	V3LHS_638999
pGIPZ scramble shRNA	Dharmacon	RHS4348
Software and Algorithms		
Syngistix	PerkinElmer	N/A
Prism	GraphPad	N/A
Image StudioVersion 3.1.4	Li-Cor	N/A

Table S2. Osmotic solutions used in western blot experiments, intracellular Na^+ or K^+ concentration measurements and cell proliferation assays.

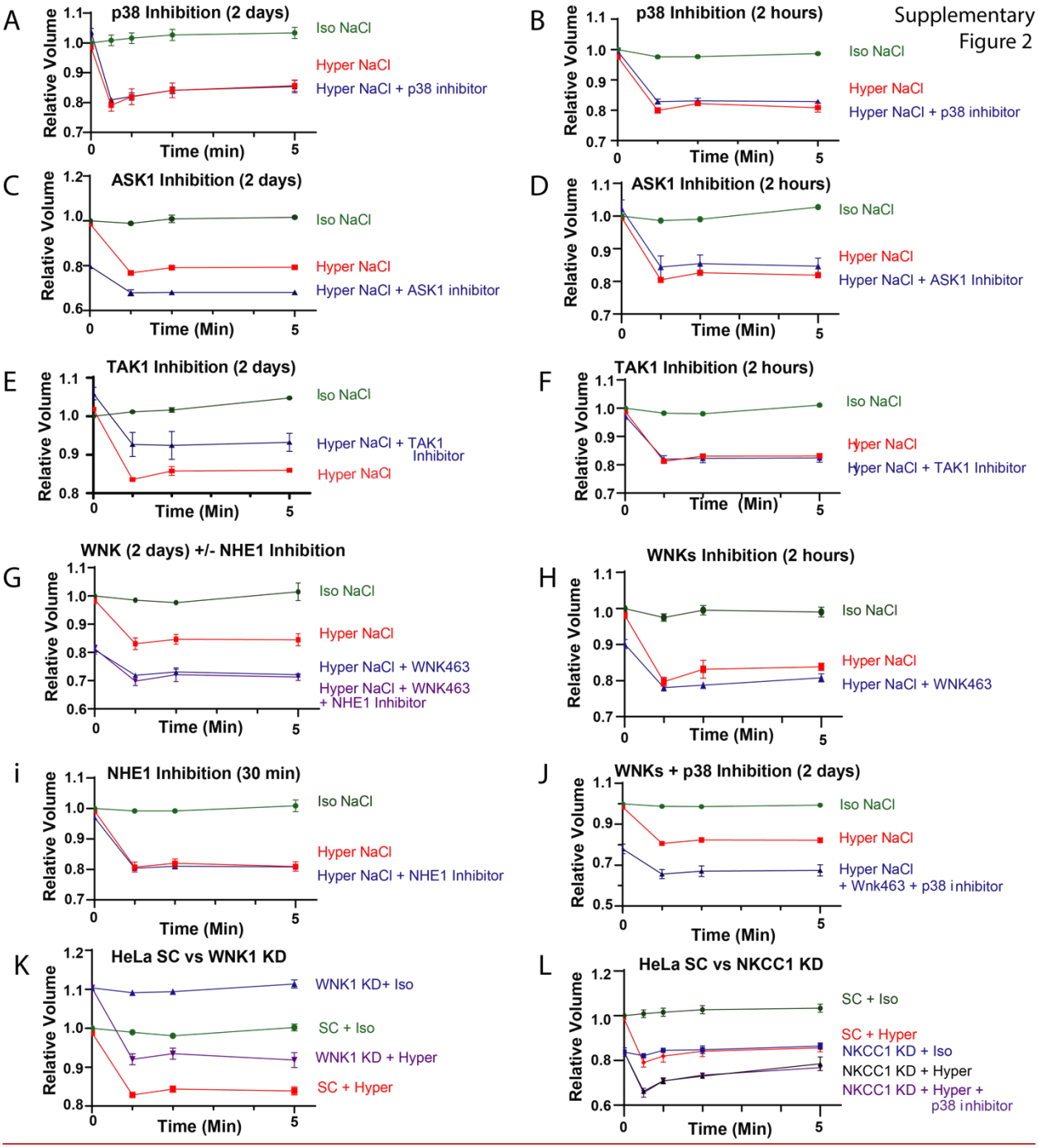
Condition	Iso-osmotic	Hyperosmotic
NaCl solutions	**315 mOsm:** 135 mM NaCl, 2.5 mM KCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4	**400 mOsm:** 180 mM NaCl, 3.33 mM KCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4 **475 mOsm:** 225 mM NaCl, 4.16 mM KCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4 **580 mOsm:** 270 mM NaCl, 5 mM KCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4
NMDG-Cl solutions	**315 mOsm:** 135 mM NMDG-Cl, 2.5 mM KCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4	**400 mOsm:** 180 mM NMDG-Cl, 3.33 mM KCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4 **475 mOsm:** 225 mM NMDG-Cl, 4.16 mM KCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4 **580 mOsm:** 270 mM NMDG-Cl, 5 mM KCl, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4
Na-Gluconate solutions	**315 mOsm:** 135 mM Na-Gluconate, 2.5mM Kgluconate, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4	**580 mOsm:** 270 mM Na-Gluconate, 5 mM K-gluconate, 0.25 mM $CaCl_2$, 0.25 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes, pH 7.4
Cellular Medium	**315 mOsm:** DMEM + 10% FBS	**475 mOsm:** DMEM + 10% FBS + 90 mM NaCl



Supplementary Figure 1. Activation of the p38 pathway by hyperosmotic or hypertonic solutions, Rac1 Inhibitor or WNK463, in different cell types.

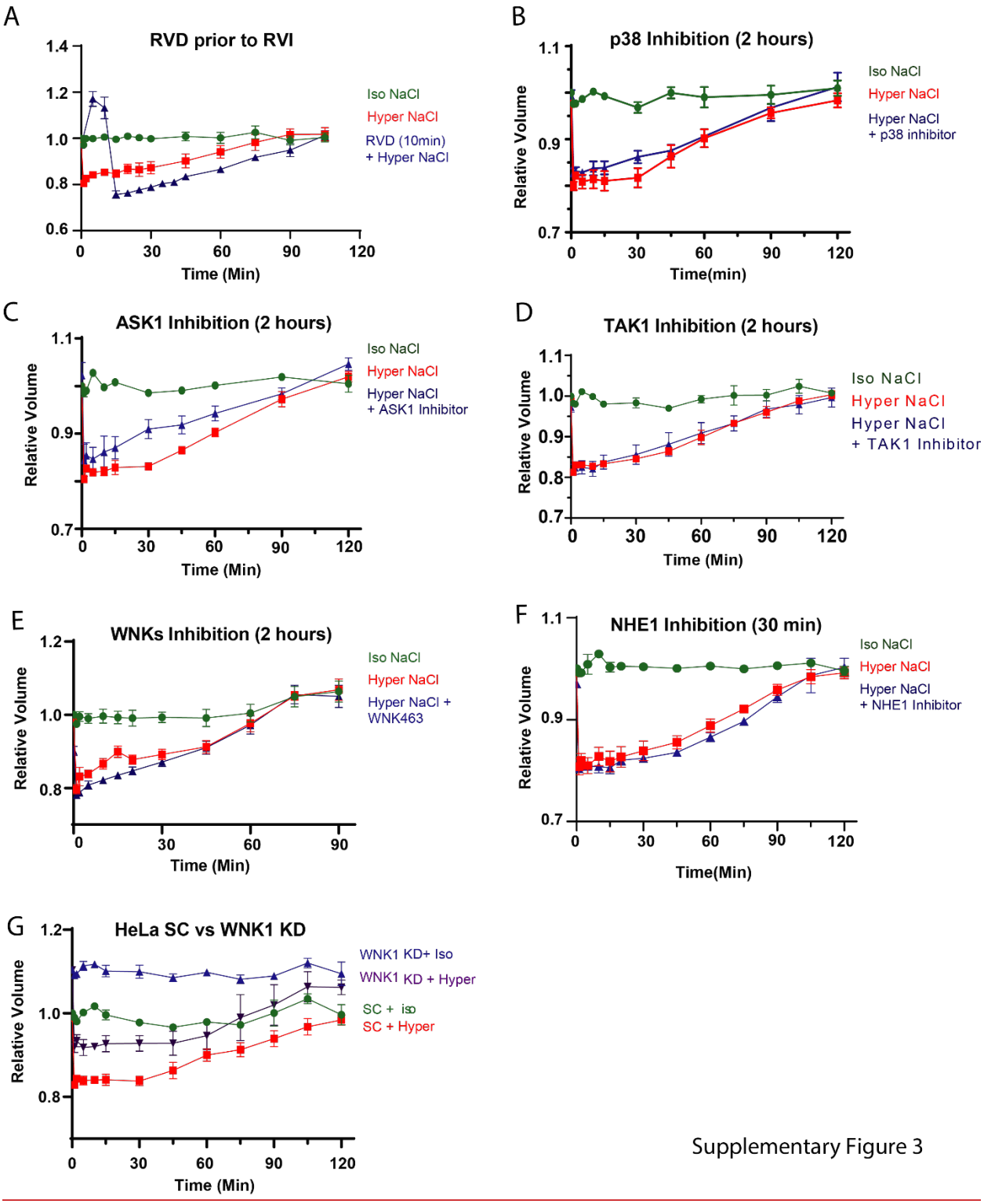
(A) HeLa cells were treated with Iso- or hyper- osmotic Choline-Cl solutions for 15 minutes. **(B)** HeLa cells were treated with an increasing concentration of NSC23766 (Rac1 inhibitor) for 2 hours followed by incubation with hyper-osmotic NaCl solution in the absence/presence of NSC23766. **(C)** HeLa cells were treated with iso-osmotic or hyper-osmotic NaCl solutions for 15 minutes. **(D)** Human Primary Bronchial Epithelial cells, or **(E)** Mouse Colonic Organoids, were treated with 10 μM WNK463 for 2 hours followed by incubation with iso- or hyper- osmotic NaCl for 15 minutes in the absence/presence of

WNK463. Quantification of active p38 (p-p38) or MKK4 (p-MKK4) is depicted below their respective blots. All data are mean±s.e.m, N≥3 independent experiments. Statistics as described in Figure 1.



Supplementary Figure 2. Cell volume changes in the first 5 minutes of the RVI experiments.

Cell volume changes in the first 5 minutes of the respective RVI experiments shown in Figures 5 and 6. p38 inhibition for **(A)** 2 days or **(B)** 2 hours; ASK1 inhibition for **(C)** 2 days or **(D)** 2 hours; TAK1 inhibition for **(E)** 2 days or **(F)** 2 hours; **(G)** WNK inhibition with or without 30 minutes NHE1 inhibition; **(H)** WNKs inhibition for 2 hours; **(I)** NHE1 inhibition for 30 minutes; **(J)** WNKs and p38 inhibition for 2 days; **(K)** HeLa scramble control (SC) or WNK1 knockdown (KD) cells; **(L)** HeLa scramble control (SC) or NKCC1 KD cells. Cell volume was measured by using a Coulter counter. All data are mean±s.e.m, N≥3 independent experiments.



Supplementary Figure 3. Regulatory Volume Increase (RVI) of HeLa cells exposed to hypo-osmotic solution before hyper-osmotic shock, or treated with short-term p38, ASK1, TAK1, WNKs or NHE1 inhibitors.

(A) HeLa Scramble control (SC) cells were treated (or not) with hypo-osmotic NaCl solution for 10 minutes prior to switching to iso- or hyper-osmotic NaCl solution to analyze RVI. (**B-E**): 2 hours inhibition prior to measuring RVI under iso- or hyper-osmotic NaCl solution in the absence/presence of: **(B)** 5 μM SB203580 (p38 inhibitor), **(C)** 10 μM GS-444217 (ASK1 inhibitor), **(D)** 1 μM 5Z-7-Oxozeaenol (TAK1 inhibitor), **(E)** 10 μM WNK463 (pan-WNKs inhibitor). **(F)** 10 μM of Cariporid (NHE1 inhibitor) applied for 30 minutes. **(G)** HeLa scramble control (SC) or WNK1 KD cells were washed with PBS and exposed to iso- or hyper-osmotic solutions for the indicated times. Cell volume recovery (RVI) was measured as above. All data are mean±s.e.m, N≥3 independent experiments.