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LRRC8A-containing chloride channel is crucial for cell volume recovery and survival under hypertonic conditions

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Figs. S1 to S10
Tables S1 and S2

Other supplementary materials for this manuscript include the following:

Tables S3 and S4 (Datasets)

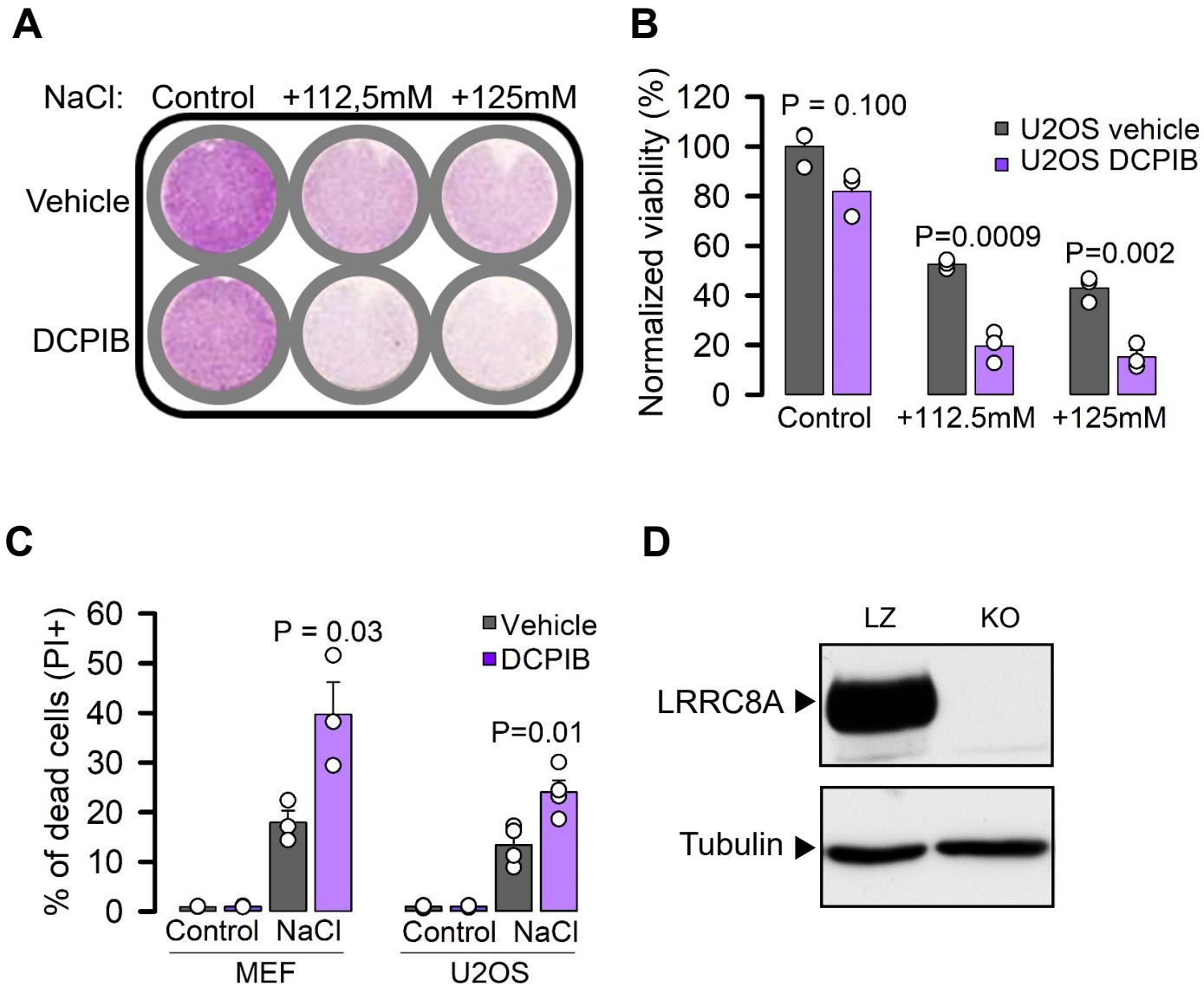

Fig. S1. Validation of LRRC8A activity requirement for survival under hyperosmolarity in U2OS and MEF cells. (A) Crystal violet staining of U2OS cells exposed to hypertonic conditions in the presence/absence of DCPIB. **(B)** Colony area quantification of crystal violet-stained U2OS cells fixed after 48 h treatment with NaCl at different concentrations in the presence/absence of DCPIB (36 μM) (n=3). **(C)** PI staining (to monitor cell death) of U2OS and MEF cells treated 24 h with NaCl (137.5 mM NaCl U2OS, 150 mM NaCl MEF) and DCPIB (36 μM) (n=3). P values shown in (b) and (c) were determined by Student's t-test. **(D)** Western blot analysis of LRRC8A obtained from extracts of LZ and KO cells.

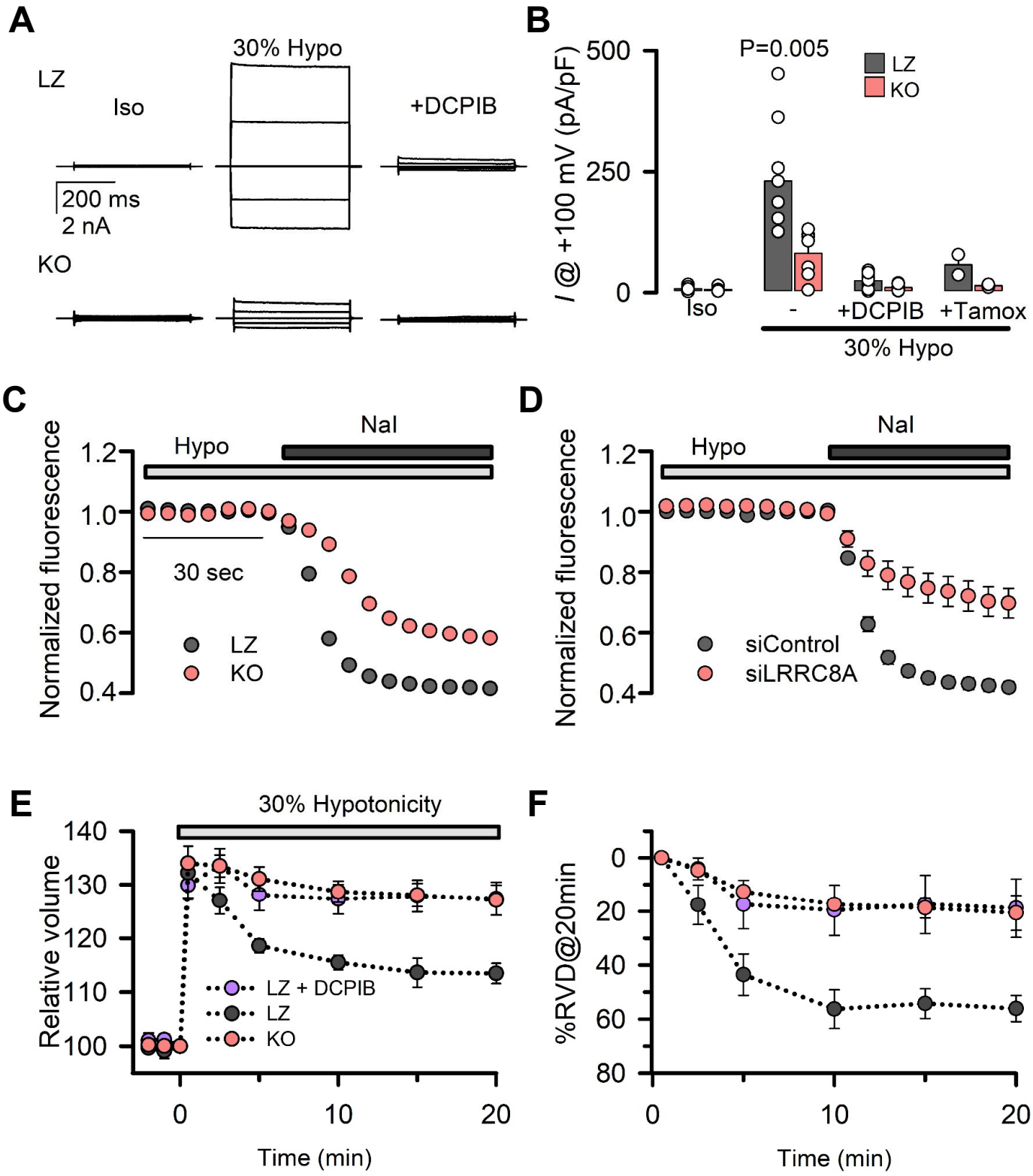

Fig. S2. Characterization of hypotonicity-induced responses. (A) Families of whole-cell chloride currents recorded in LZ and KO cells exposed to 30% hypotonic solutions followed by the addition of 36 μM DCPIB. Cells here held at 0 mV and pulsed from -100 mV to +100 mV in 50 mV steps. **(B)** Maximal mean current densities measured in LZ and KO in isotonic and hypotonic solutions with or without adding 36 μ M DCPIB or 10 μ M tamoxifen. P values comparing KO vs LZ determined by Student's t-test. **(C, D)** Mean ±SEM YFP fluorescence change (normalized to the baseline in isotonic conditions) in LZ (n=71) and KO (n=67) cells (c) or control siRNA (n=21) and siLRRC8A-treated (n=22) HeLa cells (d). Addition of hypotonic bathing solutions containing NaCl or NaI is indicated by boxes at the top of recordings. **(E)** Time course of relative changes in cell volume (normalized to isotonic conditions) before and after exposure of LZ and KO cells to a 30% hypotonic medium. Mean ±SEM (n=6). **(F)** Percentage of RVD, calculated as the percentage of volume recovered following the initial cell swelling at the different time points after the hypotonic stress.

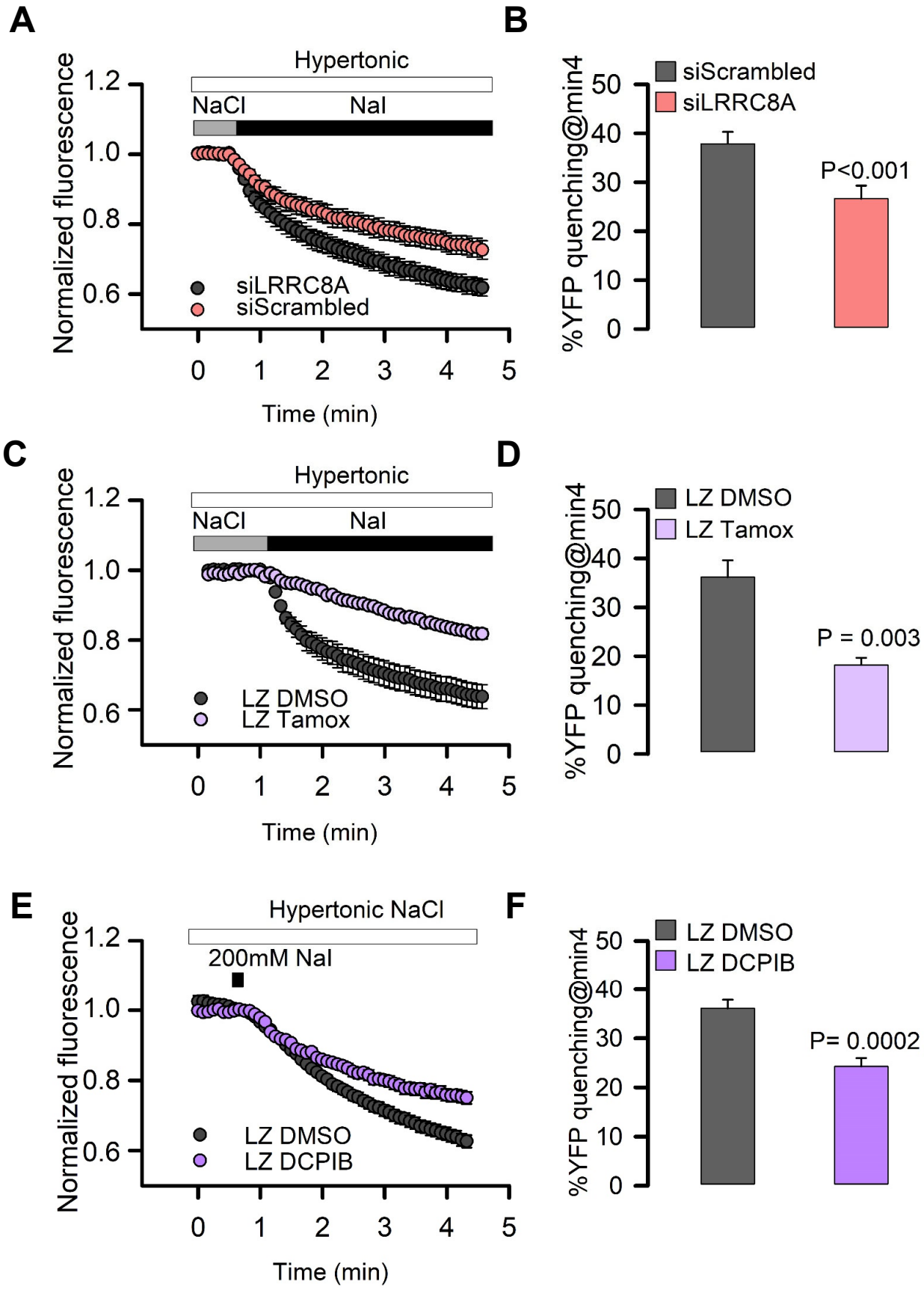

Fig. S3. Hyperotonicity-induced YFP quenching. (A) Mean±SEM YFP fluorescence change (normalized to the baseline in NaCl containing solutions). **(B)** Percentage of YFP quenching (right) measured 4 min after the addition of NaI in Scrambled siRNA (n=46) or LRRC8A siRNA (n=48) transfected HeLa cells also expressing the halide-sensitive YFP. **(C)** Mean ±SEM YFP fluorescence change (normalized to the baseline in NaCl containing solutions). **(D)** Percentage of YFP quenching (right) measured 4 min after the addition of NaI in LZ cells treated with DMSO (n=51) or 10 μM tamoxifen (n=23). **(E)** Mean ±SEM YFP fluorescence change (left, normalized to the baseline in NaCl containing solutions). **(F)** Percentage of YFP quenching measured 4 min after the addition of NaI in LZ cells treated with DMSO (n=63) or 36 μM DCPIB (n=26). P values determined by two-tailed Student's t-test.

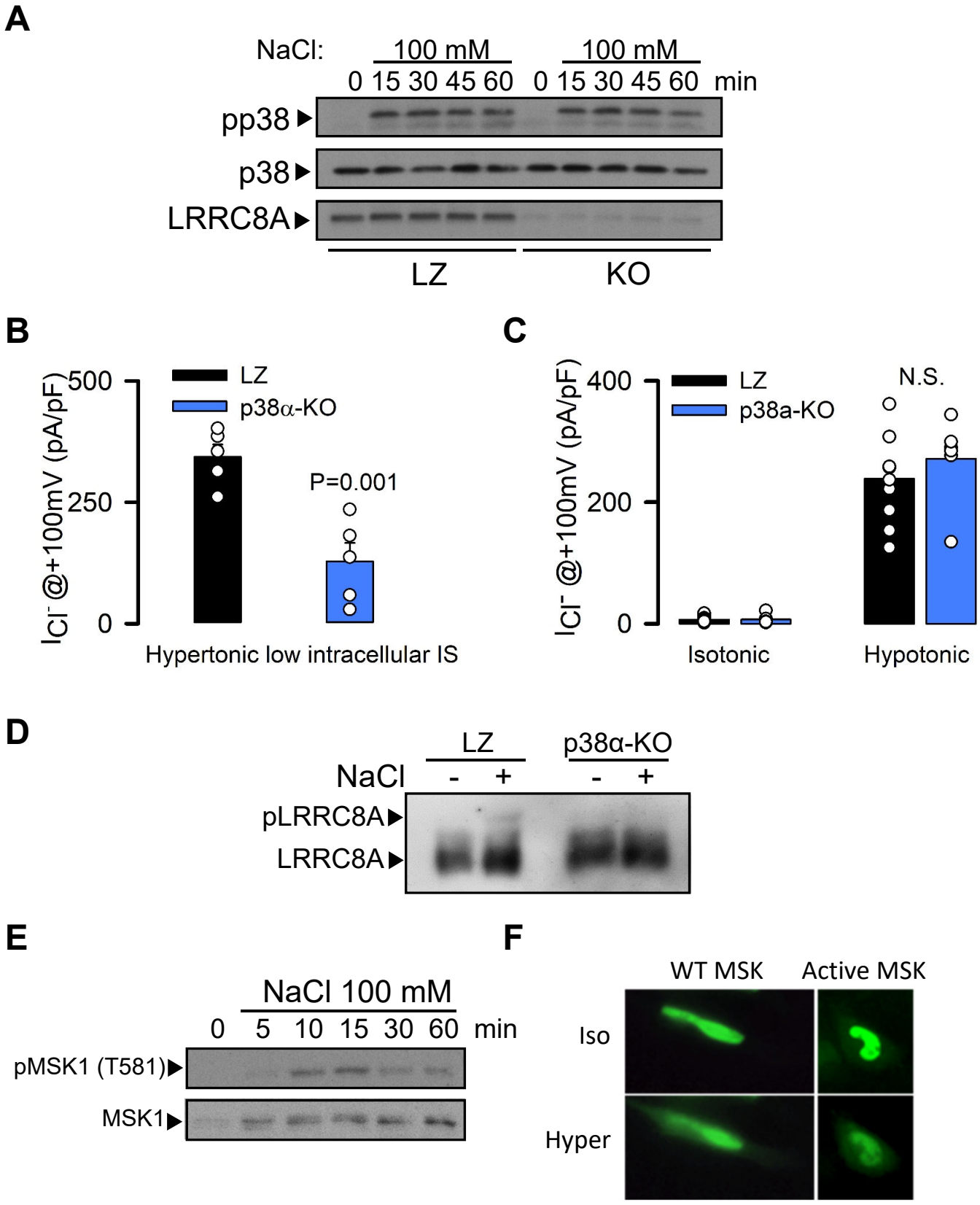

Fig. S4. p38/MSK1 pathway modulates LRRC8A activity. (A) Western blot analysis of p38 activation under hypertonic stress in extracts obtained from HeLa LZ and KO cells **(B)** Maximal mean current densities measured in LZ (n=5) and p38α-KO (n=5) HeLa cells dialyzed with hypertonic solutions of 0.08 IS and exposed to hypertonic conditions. **(C)** Maximal mean current densities measured in LZ (n=13) and p38α-KO (n=6) in isotonic and hypotonic solutions. P values determined by two-tailed Student's t-test. **(D)** Western blot analysis of LRRC8A in a phostag gel of extracts obtained from HeLa WT and p38α-KO cells exposed to hypertonic solutions (+100 mM NaCl, 15 min) **(E)** Western blot analysis of MSK1 activation under hyperosmotic stress of extracts obtained from HeLa cells. **(F)** Immunofluorescence images showing the localization of GFP-tagged MSK1-WT and MSK1-T581D/T700D (active) under isotonic and hypertonic conditions.

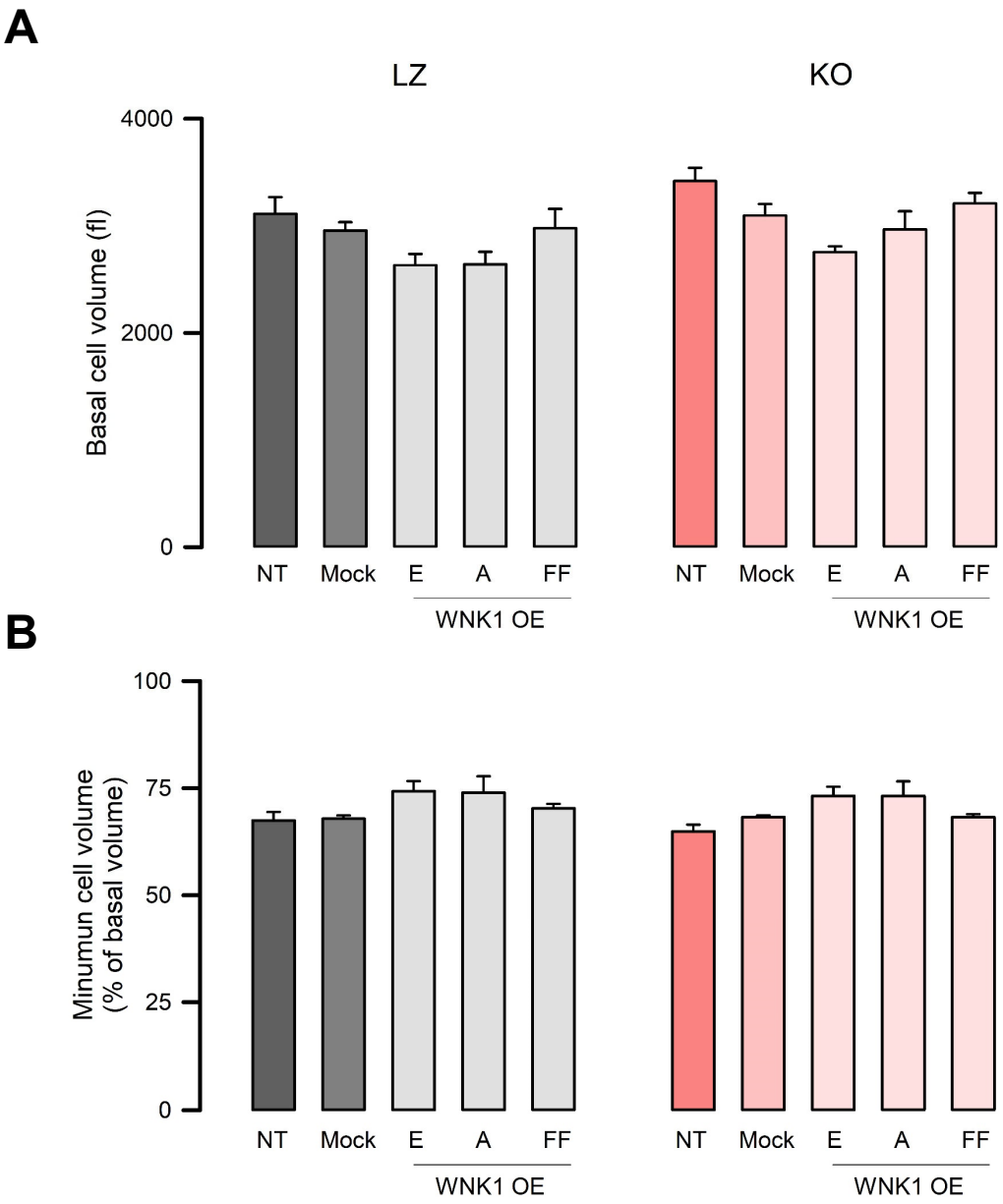

Fig. S5. Basal cell volume and peak relative shrinkage. (A) Basal cell volume of LZ and KO cells transiently overexpressing mock or different WNK1 plasmids. Mean ± SEM of 6 LZ, 7 LZ mock, 5 LZ WNK1-E, 7 WNK1-A, 5 LZ WNK1-FF, 6 KO, 5 KO mock, 11 KO WNK1-E, 4 WNK1-A and 7 WNK1-FF. **(B)** Peak relative cell shrinkage of cells under the conditions described in A. No statistical differences were observed in either basal cell volume or peak shrinkage between mock transfected cells and any other condition using ANOVA followed by Dunnett's post hoc test.

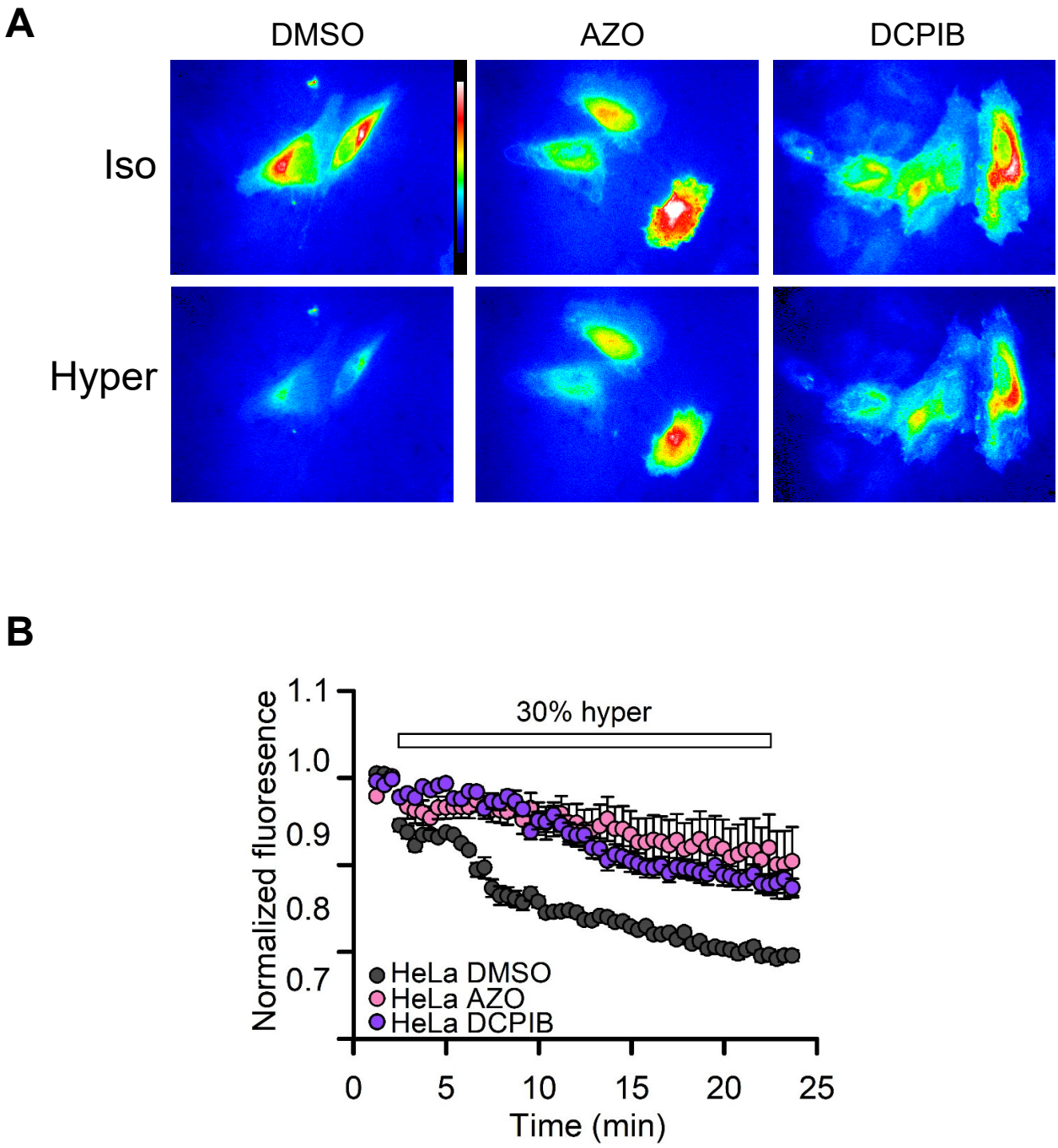

Fig. S6. LRRC8A blocker reduces NKCC1 activity. (A) Fluorescence Images of cells transfected with YFP-tagged NKCC1 and exposed to isotonic and hypertonic solutions (30 min). Note the reduction of YFP fluorescence signal under control (DMSO) hypertonic conditions, whereas smaller reduction was observed in cells treated with 36 μM DCPIB or the NKCC1 inhibitor, azosemide (10 μM). **(B)** Mean ±SEM YFP fluorescence change (normalized to the baseline in isotonic conditions) in NKCC1-YFP–expressing HeLa cells exposed to hypertonicity in the presence of DMSO, azosemide or DCPIB.

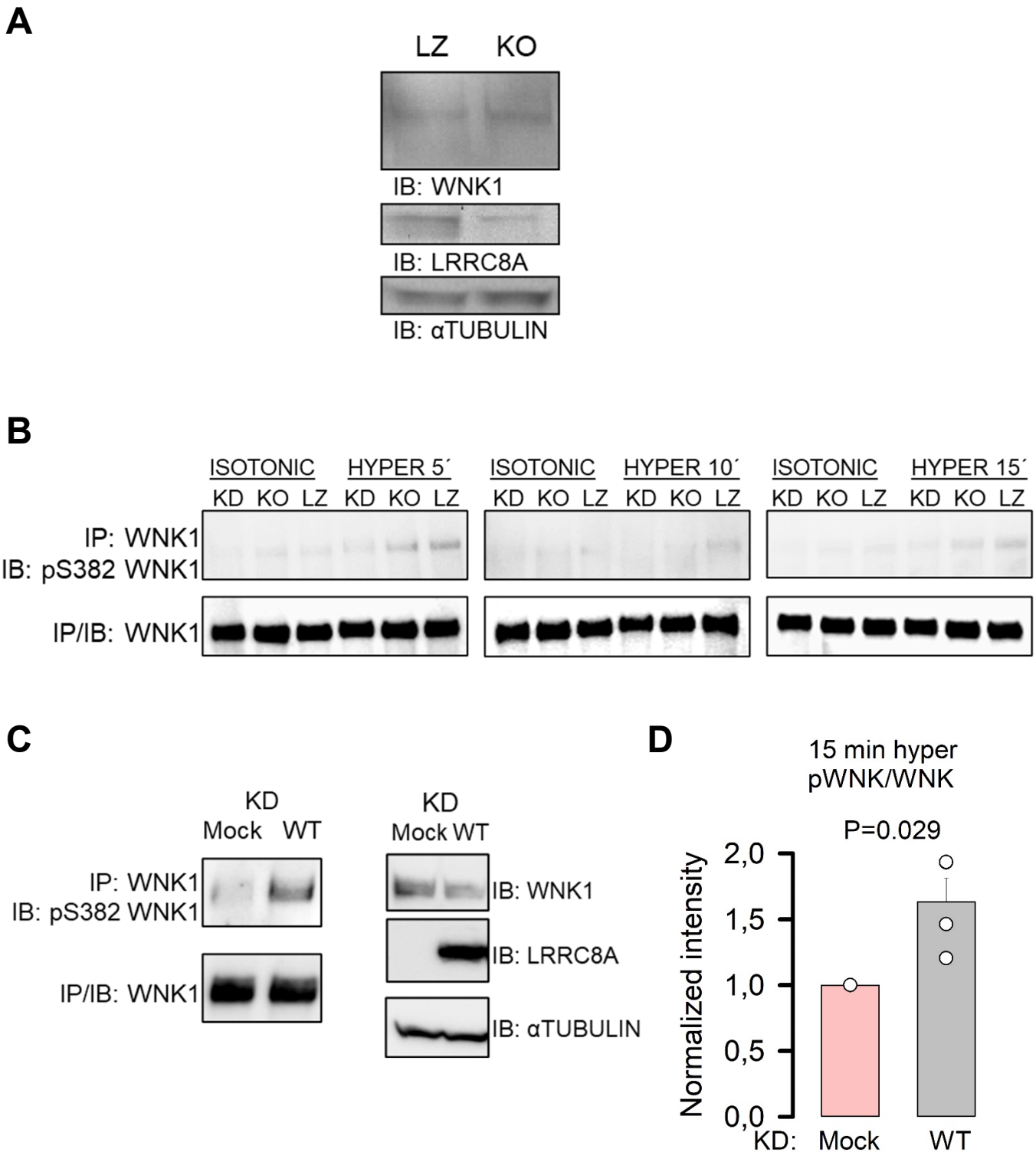

Fig. S7. LRRC8A triggers WNK activation to promote RVI and cell survival under hypertonic conditions. (A) Western blot of inputs corresponding to IP of Fig. 4a. **(B)** Phosphorylation of immunoprecipitated WNK1 obtained from cell lysates of LZ, KO and KD HeLa cells exposed to isotonicity and after 5, 10 and 15 min in 30% hypertonic medium. **(C)** Phosphorylation of immunoprecipitated WNK1 obtained from cell lysates of KD HeLa cells transfected with mock or shRNA-resistant LRRC8A and exposed to 30% hypertonic medium. Left, Western blot of p-S382 and total WNK1. Right, western blot of the inputs corresponding to the IP shown. **(D)** Quantification of phosphorylated WNK1 (normalized to total immunoprecipitated WNK1) after 15 min exposure to hypertonic medium. *P* value were determined by Mann-Whitney rank sum test (n = 4).

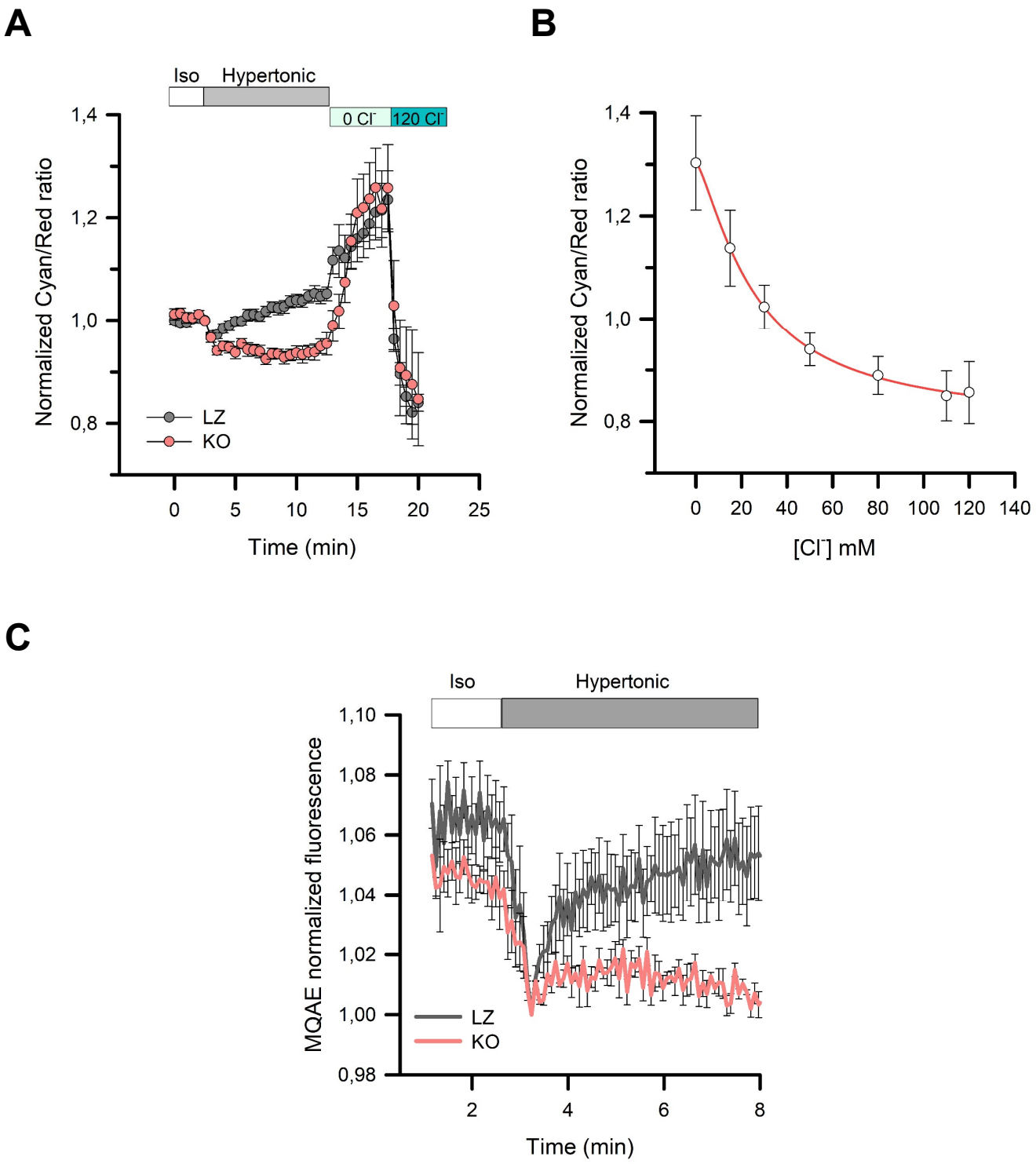

Fig. S8. Intracellular [Cl-] in response to hypertonic conditions. (A) Mean±SEM relative changes in intracellular Cl^- concentration measured using the ClopHensor are represented as the ratio of the cyan excitation (Cl^- sensitive) to red excitation (Cl^- insensitive) in different intact cells. Addition of 30% hypertonic bathing solutions and calibrating solutions containing 0Cl or 120 mM Cl is indicated by boxes at the top of recordings. Calibration solutions contained tributyltin chloride and nigericin to permeabilize cell membrane to Cl^- and equilibrate the intracellular Cl^- concentration with that present in the bathing solution. LZ, n=23; KO, n=20 from two independent trasnfections. **(B)** Intracellular Cl^- calibration of ClopHensor in LZ cells (n=7) under isotonic conditions using bathing solutions containing 0, 15, 30, 50, 80, 110 y 120 mM Cl-. Red line was drawn using a sigmoidal logistic 4 parameter function. **(C)** Intracellular [Cl^-] measured in cells loaded with the Cl^- probe MQAE. Normalized mean±SEM fluorescence change obtained from LZ (n=5) and KO (n=4) experiments (number of cells/experiment ≥40) exposed to 30% hypertonic solutions.

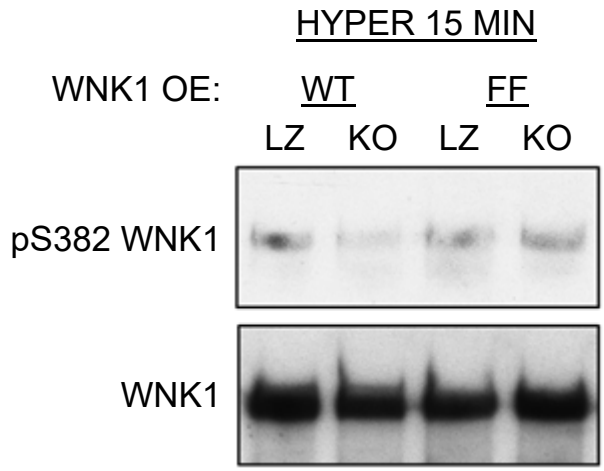

Fig. S9. Phosphorylation of WNK1 overexpressd in LZ and KO cells. Phosphorylation of WNK1-WT and WNK1-FF expressed in LZ and KO HeLa cells exposed to 30% hypertonic medium. Top, western blot of p-S382-WNK1; bottom, western blot of total WNK1.

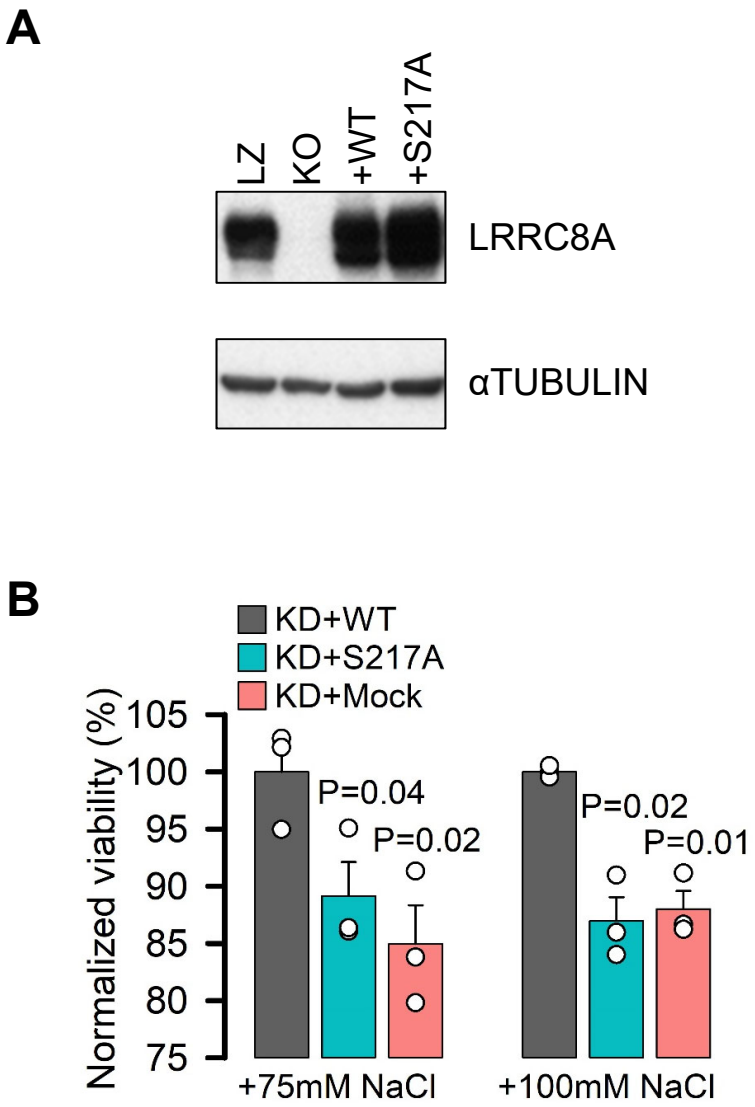

Fig. S10. LRRC8A expression in inducible cells and cell survival. (A) Western blot of LRRC8A in control, KO and inducible WT and S217A cell lines in the presence of 60 ng/ml doxycycline. **(B)** Crystal violet staining to monitor cell survival of KD HeLa cells overexpressing shRNA-resistant LRRC8A-WT or LRRC8A-S217A exposed to different doses of hyperosmotic stress. P values determined by ANOVA followed by Dunnett's post hoc test versus KD-WT group.

Table S1. Top 50 genes that are essential for cell survival upon hypertonicity identified by an unbiased loss-of-function genetic screen using the CRISPR-Cas9 system in HeLa cells.

Gene Symbol	Gene Rank	RRA	FDR	Gene name
LRRC8A	1	1.8692E-07	0.064356	Leucine-rich repeat-containing protein 8A
AGPAT5	2	0.000014151	0.648515	1-Acylglycerol-3-Phosphate O-Acyltransferase 5
CTRB1	3	0.000014276	0.54703	Chymotrypsinogen B1
CCDC85C	4	0.000033069	0.873762	Coiled-Coil Domain Containing Protein 85C
AIPL1	5	0.000041066	0.873762	Aryl Hydrocarbon Receptor Interacting Protein Like 1
NEURL1B	6	0.000051732	0.873762	Neuralized E3 Ubiquitin Protein Ligase 1B
LPIN3	7	0.000052284	0.873762	Lipin 3
SULF2	8	0.000053273	0.873762	Sulfatase 2
BAIAP3	9	0.000071128	0.873762	Brain-Specific Angiogenesis Inhibitor I-Associated Protein 3
ZNF646	10	0.000088054	0.873762	Zinc Finger Protein 646
HAP1	11	0.000090202	0.873762	Huntingtin Associated Protein 1
GJC2	12	0.000099141	0.873762	Gap Junction Protein Gamma 2 / Connexin-46.6
TPM1	13	0.00010278	0.874072	Tropomyosin 1
IGFBP7	14	0.00010417	0.874072	Insulin Like Growth Factor Binding Protein 7
ATCAY	15	0.00013271	0.87698	ATCAY Kinesin Light Chain Interacting Caytaxin
CABP1	16	0.00013321	0.87698	Calcium Binding Protein 1
VCAM1	17	0.00013378	0.87698	Vascular Cell Adhesion Molecule 1
CPA1	18	0.00015472	0.911427	Carboxypeptidase A1
CYP1B1	19	0.0001656	0.911427	Cytochrome P450 Family 1 Subfamily B Member 1
DACT3	20	0.00017027	0.911427	Dishevelled Binding Antagonist Of Beta Catenin 3
CCDC28B	21	0.0001713	0.911427	Coiled-Coil Domain-Containing Protein 28B
EXD3	22	0.00018027	0.911427	Exonuclease 3'-5' Domain Containing Protein 3
PSMD10	23	0.00020496	0.873762	Proteasome 26S Subunit, Non-ATPase 10
RNPEP	24	0.00021395	0.911427	Arginyl Aminopeptidase
SPTSSA	25	0.00021465	0.873762	Serine Palmitoyltransferase Small Subunit A
TICAM1	26	0.00021533	0.911427	Toll Like Receptor Adaptor Molecule 1
GAS2	27	0.00022022	0.911427	Growth Arrest Specific 2
MYBL2	28	0.00023073	0.911427	Myb-Like Protein 2
THSD4	29	0.00023981	0.911427	Thrombospondin Type-1 Domain-Containing Protein 4
HOOK2	30	0.00024476	0.911427	Hook Microtubule Tethering Protein 2
NEXN	31	0.00025273	0.87698	Nexilin F-Actin Binding Protein
DUSP21	32	0.00025979	0.911427	Dual Specificity Phosphatase 21
SLC5A6	33	0.00029145	0.911427	Solute Carrier Family 5 Member 6
LAMP2	34	0.00029688	0.911427	Lysosomal Associated Membrane Protein 2
IMPAD1	35	0.00030832	0.911427	Inositol Monophosphatase Domain-Containing Protein 1
CRTAC1	36	0.00032536	0.911427	Cartilage Acidic Protein 1

TMEM132C	37	0.00033798	0.911427	Transmembrane Protein 132C
HES3	38	0.00033998	0.911427	Hes Family BHLH Transcription Factor 3
MXRA5	39	0.00036572	0.911427	Matrix-Remodeling-Associated Protein 5
HSPB6	40	0.00037682	0.911427	Heat Shock Protein Beta-6
KIAA1967	41	0.00038812	0.911427	Cell Cycle And Apoptosis Regulator Protein 2
RGS19	42	0.00040146	0.911427	Regulator Of G Protein Signaling 19
SCGB3A1	43	0.00041873	0.911427	Secretoglobin Family 3A Member 1
CNR1	44	0.00042211	0.911427	Cannabinoid Receptor 1
FKRP	45	0.00042282	0.911427	Fukutin Related Protein
C3orf36	46	0.00045652	0.911427	Chromosome 3 Putative Open Reading Frame 36
SIL1	47	0.00046036	0.911427	SIL1 Nucleotide Exchange Factor
GPBP1	48	0.00046932	0.911427	GC-Rich Promoter Binding Protein 1
PRG2	49	0.00047606	0.911427	Proteoglycan 2
ROBO4	50	0.00047644	0.911427	Roundabout Guidance Receptor 4

Table S2. Drugs, antibodies, oligonucleotides and plasmids used in this work.

DRUGS; SUPPLIER	**TARGET**	**[µM]**	**INCUBATION**	**VEHICLE**
DCPIB; Tocris	LRRC8A, inhibits	37.5	Acute (patch), preincubated 15 min (volume, imaging)	DMSO
Tamoxifen; Sigma	LRRC8A, inhibits	10	Acute (patch), preincubated 15 min (imaging)	Ethanol
SB203580; Calbiochem	p38, inhibits	10	Preincubated 45-60 min (patch, volume)	DMSO
SB747651A; Tocris	MSK1/2, inhibits	10	Preincubated 45-60 min (patch, volume)	DMSO/H_2O
Bumetanide; Sigma	NKCC1, inhibits	50	Preincubated 30 min (volume)	DMSO
Azosemide; TRC	NKCC1, inhibits	25	Preincubated 30 min (volume)	DMSO
6-methoxyquinolyl) acetoethyl ester (MQAE); ThermoFisher	[Cl^-] fluorescent probe	5000	60 min	DMSO
Tributyltin chloride; Sigma	Cl^-/OH^- ionophore	10	acute	DMSO
Nigericin; ThermoFisher	K^+/H^+ exchange	5	acute	methanol
ANTIBODIES	**REFERENCE**	**[µM]**		
Rabbit polyclonal anti-LRRC8A	#A304-175A-M Bethyl (Bionova)	1:1000 (5% BSA)		
Mouse monoclonal anti-LRRC8A	SAB1412855-100U Sigma	1:1000 (5% BSA)		
Anti-p38α	#9228 Cell signaling	1:1000 (5% BSA)		
Anti-phospho-p38α	#9215 Cell signaling	1:1000 (5% BSA)		
anti-MSK1	#3489 Cell signaling	1:1000 (5% BSA)		
anti-phospho-MSK1 (Thr581)	#9595 Cell signaling	1:1000 (5% BSA)		
Mouse monoclonal anti-α-tubulin	#T6074 (Sigma)	1:5000 (5% MILK)		

Sheep anti-WNK1 total	S650C https://mrcppureagents.dundee.ac.uk/	1-2 µg/ml (5% MILK)
Sheep anti-pS382-WNK1	S099B https://mrcppureagents.dundee.ac.uk/	2 µg/ml (5% MILK) incubated in the presence of 10 µg/ml of a dephosphorylated form of phosphopeptide antigen.
Secondary anti-rabbit HRP	NA934, GE healthcare	1:2000 (5% MILK)
Secondary anti-mouse HRP	NXA931, GE healthcare	1:5000-1:10000 (5% MILK)
Secondary anti-sheep HRP	#31480, Invitrogen	1:5000-1:10000 (5% MILK)

PLASMIDS	**REFERENCE**
SWELL1-pIRES2-EGFP-RNAi-Resistant WT	Provided by Dr. A. Patapoutian (The Scripps Research Institute, La Jolla, USA)
pcDNA3.1-EYFP-H148Q/I152L	Provided by Dr. L Galietta (University of Naples Federico II, Naples, Italy.)
pEGFP-C2-MSK1 T581/D+T700D	DU6268 https://mrcppureagents.dundee.ac.uk/
pEGFP-C1-FLAG-MSK1- WT	DU6445 https://mrcppureagents.dundee.ac.uk/
pEBG-2T-FLAG-GST full WNK1-S382/E	DU6851 https://mrcppureagents.dundee.ac.uk/
pEBG-2T- FLAG-GST full WNK1-S382/A	DU6850 https://mrcppureagents.dundee.ac.uk/
pEBG FLAG GST GST WNK1-L369F /L371F	Generated from plasmid DU687008 https://mrcppureagents.dundee.ac.uk/
pEBG FLAG GST WNK1-WT	DU562119 https://mrcppureagents.dundee.ac.uk/
pcDNA3.1 Flag Cl-sensitive YFP hNKCC1 WT (NT13)	#49060 Addgene
pcDNA3.1ClpHensor	Provided by Dr. P. Bregestovski (Inserm UMR1106, Aix-Marseille University, Marseille, France)

MOLECULAR BIOLOGY TOOLS	**REFERENCE**	**TARGET SEQUENCE**
siRNA LRRC8A	Dharmacon (Cat#J-026211-09-0020	GGUACAACCACAUCGCCUA

OLIGONULEOTIDES	**SEQUENCE**
gRNA sequence targets	
LacZ-fwd	ACCGCCCGAATCTCTATCGTGCGG
LacZ-rvs	AAACCCGCACGATAGAGATTCGGG
LRRC8A-SC-GD1-fwd	ACCGTGTGTTTGACCTGGTGGAGC
LRRC8A-SC-GD1-rvs	AAACGCTCCACCAGGTCAAACACA
LRRC8A-SC-GD2-fwd	ACCGAGCCGCTCCTTCACCTCGGG
LRRC8A-SC-GD2-rvs	AAACCCCGAGGTGAAGGAGCGGCT
p38α -fwd	ACCGAGCTCCTGCCGGTAGAACGT
p38α-rvs	AAACACGTTCTACCGGCAGGAGCT
MSK1 putative phosphorylation sites in LRRC8A	
S151A	GCG CAC CAG CGC GAA GCT GGA GC
	GGG AAT TTGA ACC AGA AGT TGC TGC AGG
S174A	GAG GGC CCT GGC GGA GAC AGT GG
	GTG GTC CAG GGC GAG TCG AAG

S217A	GCG GAC CAA GGC ACG GAT CGA GC
	TGC AGC ATG GGC ACG GTG
S217E	GCG GAC CAA GGA ACG GAT CGA GCA G
	TGC AGC ATG GGC ACG GTG
T229A	CCG CTC AGA GGC GGG CGT GCT GG
	TCC ACG ATA CCC TGC TCG ATC CGT G
LRRC8A ICL loop fragment - pGEX-6P-1	
	ATG AAT TCA AAT TCC CGC GCA CCA GCT CGA
	ATG CGG CCG CGT CCC CCT CCT CCA CAT GGG TC
LRRC8A full length - pGEX-6P-1	
	ATG AAT TCA TGG AAC AAA AAC TTA TTT CTG AAG AAG ATC TGA TTC CGG TGA CAG AGC TCC GCT ACT TTG CG
	ATG CGG CCG CTC AGG CCT GCT CCT TGT CAG CC
MSK1 - pETM11	
	ATA TAT CCA TGG CGA GGA GGA GGG TGG CAG CAG CG
	ATA TAT GCG GCC GCA GCT ACT GAG TCC GAG AAC TG
LRRC8A - pSB-BN	
	AAT ATG GCC TCT GAG GCC ACC ATG ATT CCG GTG ACA GAG CTC CG
	AAT ATG GCC TGA CAG GCC TCA GGC CTG CTC CTT GTC AGC CCT CCA TAA TCT TTC TTT TAC TTC AGG TGG CAG TGT G

Table S3. Quality control of the screening. Gene Set Enrichment Analysis (GSEA) of KEGG pathways of MAGeCK ranked genes for two independent comparisons. Essential genes are depleted eight days after the establishment of the sgRNA library infection. Gene Set Enrichment Analysis (GSEA) of KEGG pathways of MAGeCK ranked genes for two independent comparisons. Control sheet: Day 8 control samples compared to Day 0; NaCl sheet: Day 8 NaCl treated samples compared to Day 0. Pathway is the KEGG category name. Size is the total number of genes in this pathway. ES is the Enrichment Score of the GSEA. p is the p-value calculated by GSEA. p_permutation is the p-value calculated by permutation test. FDR correction of the p-value. Ranking is the order of the pathways based on the ES. Hits are the number of genes below the considered cutoff.
See in https://figshare.com/s/647e734e484ca4e8685b

Table S4. MAGeCK results for hyperosmotic stress screening analysis. Identification of fitness genes done with MAGeCK's "test" command. Id is the HGNC gene symbol. Gene_Description is the WikiGene description of the gene. Num is the total number of sgRNAs targeting the gene. Neg.score is the RRA score. Neg.p.value is the p-value computed by MAGeCK's permutation algorithm. Neg.fdr is the FDR multiple testing correction computed from p-values. Neg.rank is the ranking of the gene according to RRA. Neg.goodsgrna is the number of sgRNAs that pass the FDR threshold for gene testing (default 0.25). neg.lfc is the gene log fold change (lfc) computed as the average of sgRNAs lfc.
See in https://figshare.com/s/6c1d4631a16847d09bf3