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

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Regulation of cell volume is essential for tissue homeostasis and cell viability. In response to hypertonic stress, cells need rapid electrolyte influx to compensate water loss and to prevent cell death in a process known as regulatory volume increase (RVI). However, the molecular component able to trigger such a process was unknown to date. Using a genome-wide CRISPR/Cas9 screen, we identified LRRC8A, which encodes a chloride channel subunit, as the gene most associated with cell survival under hypertonic conditions. Hypertonicity activates the p38 stress-activated protein kinase pathway and its downstream MSK1 kinase, which phosphorylates and activates LRRC8A. LRRC8A-mediated Cl− efflux facilitates activation of the with-no-lysine (WNK) kinase pathway, which in turn, promotes electrolyte influx via Na+/K+/2Cl− cotransporter (NKCC) and RVI under hypertonic stress. LRRC8A S217A mutation impairs channel activation by MSK1, resulting in reduced RVI and cell survival. In summary, LRRC8A is key to bidirectional osmotic stress responses and cell survival under hypertonic conditions.

LRRC8A chloride channel | NKCC | p38/MSK1 | RVI | osmostress

The regulation of cell volume is essential for organism homeostasis (1). Cell swelling or shrinkage following osmotic stress exerts profound alterations of the cellular status (2), from short-term changes in the concentration of cell contents and signaling molecules (3) to modifications of the cell cycle (4, 5), transcription (6, 7), and chromatin organization (8). If not restored, such changes may cause cell death and pathological conditions, including neurological and epithelial alterations (9, 10). Cells have developed various mechanisms to rapidly regulate their volume when exposed to conditions that cause their shrinkage or swelling, the so-called regulatory volume increase (RVI) and regulatory volume decrease (RVD), respectively (1). When exposed to hypertonic environments, cells rapidly operate RVI mechanisms to preserve their functions and tissue survival (11). RVI involves electrolyte influx via the Na+/K+/2Cl− cotransporter (NKCC) (12–15). NKCC activation was early associated to a reduced intracellular [Cl−] (12, 14, 15) and the phosphorylation of the transporter (16). Later on, the role of the chloride-sensitive with-no-lysine (WNK) kinase pathway and its downstream targets in the activation of NKCC was identified (17–20). Na+/H+ and Cl−/HCO3− exchangers working in parallel also cooperate to RVI (21). On the other hand, RVD mainly involves ion efflux through Cl− (22, 23) and K+ channels (24, 25), which respond to different intracellular signaling or mechanical cues triggered by cell swelling, or via K+/Cl− cotransporters also modulated by WNK (26). Therefore, most of the effectors participating in the regulation of cell volume are identified, but less is known about the mechanisms that initiate RVI. Recent evidence suggests direct osmosensing by the kinase domain of WNK due to a displacement of the equilibrium from a chloride binding conformation (inhibitory) to a low water availability (activating) conformation under high osmolarity conditions (27). In this model, reduction of the intracellular [Cl−] may facilitate hyperosmotic-dependent activation of WNK and RVI, in line with reports from the early 1990s pointing to the link between low intracellular [Cl−] and osmotically activated NKCC transport (12, 15). However, despite an intense search for the molecular pathways that regulate Cl− movement across the plasma membrane when challenged by osmotic changes, there is still no answer for a question presented more than 30 y ago: How do cells maintain intracellular Cl− at levels that facilitate WNK/NKCC activation and engage an effective RVI to protect them from death under hypertonic stress?

Results

LRRC8A Chloride Channel Favors Cell Survival under Hypertonic Conditions. To identify genes that are essential for cell survival upon hypertonicity, we conducted an unbiased loss-of-function genetic screen using the CRISPR-Cas9 system in HeLa cells infected with two libraries, each one containing around 90,000 guide RNAs (gRNAs) (28). Cells were grown for 6 d to discard essential genes. Then, cells were

Significance

Rapid regulatory volume increase (RVI) is important for cell survival under hypertonic conditions. RVI is driven by Cl− uptake via the Na+/K+/2Cl− cotransporter (NKCC), which is activated by WNK kinases following a reduction in intracellular [Cl−]. However, how intracellular [Cl−] is regulated to modulate the WNK–NKCC axis and engage a protective RVI remains unknown. Our work reveals that LRRC8A-containing chloride channel is a key protective factor against hypertonic shocks. Considering that LRRC8A (SWELL1) is typically activated by low ionic strength under hypotonic stress, our results posed another interesting question: what activates this chloride channel under hypertonic stress? We demonstrated that, upon hyperosmotic activation, the p38/MSK1 pathway gates LRRC8A-containing chloride channel to facilitate activation of WNK–NKCC and an effective RVI.

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subjected to hypertonic conditions for 24 h (+150 mM NaCl) (Fig. 1A). LRRC8A, which encodes for a subunit of a swelling-activated chloride channel (29, 30), was the gene most associated with increased hypertonicity-induced cell death (Fig. 1B and SI Appendix, Table S1 and Fig. S4). The use of a crystal violet assay or propidium iodide (PI) staining to quantify cell viability in several cell lines exposed to the specific LRRC8A channel inhibitor 4-(2-buty1-6,7-dichloro-2-cyclopentylmethyl-1-ox-5-yl)oxybutyric acid (DCPIB) (31) confirmed the role of LRRC8A in cell survival under hypertonic conditions (Fig. 1C and D and SI Appendix, Fig. S1A–C). Notably, cell death almost doubled in those cells exposed to DCPIB and extracellular hypertonicity. The involvement of LRRC8A in the protection against hypertonicity-induced cell death was also validated in HeLa-LRRC8A-KO (knock-out) cells engineered using the CRISPR technique (Fig. 1D and SI Appendix, Fig. S1D).

The LRRC8A Cl\(^{-}\) channel is activated in response to low ionic strength (IS) following cell swelling (32–34). We tested whether LRRC8A preserved this function in our HeLa model. A swelling-activated Cl\(^{-}\} current with higher permeability to I\(^{-}\) than to Cl\(^{-}\) has been reported in HeLa cells (35). In KO and HeLa cells transfected with small interfering RNA (siRNA) against LRRC8A, the activity of a swelling-activated chloride channel sensitive to DCPIB and tamaoxifen (36) was reduced compared to control LacZ cells (LZ), as shown by electrophysiological experiments (Fig. S2A and B), quenching of the fluorescence signal caused by I\(^{-}\) influx into cells expressing the halide-sensitive yellow fluorescent protein (YFP) (29, 30, 37) (SI Appendix, Fig. S2 C and D), and RVD analysis (SI Appendix, Fig. S2 E and F). These observations thus confirm the defined role of LRRC8A as a necessary subunit of the swelling-activated chloride channel in these cells.

Our finding that LRRC8A, in addition to its role in the cellular response to swelling, is also involved in protecting cells against hypertonicity-induced cell death was unexpected and defied our current understanding of LRRC8A regulation by low IS and the mechanisms that cells activate to respond to volume changes. To decipher how LRRC8A is involved in the response to hypertonicity, we first measured the quenching of YFP in intact cells. Exposure to hypertonic conditions activated a plasma membrane Cl\(^{-}\) conductance, which became apparent when cells were transfected with the halide-channel substrate YFP (38–40). We hypothesized that MSK1-mediated activation of LRRC8A under hypertonic conditions provides a pathway for Cl\(^{-}\) efflux that contributes to the activation of NKCC, and therefore, we addressed how LRRC8A participates in the cell volume response to hypertonic stress. Following exposure to hypertonic stress, shrunk cells immediately react by activating a rapid uptake of electrolytes via NKCC and the osmotically obliged water, thereby regaining the initial cell volume (1). Reports on the need to reduce intracellular [Cl\(^{-}\)] to trigger NKCC activity (12, 15) and the Cl\(^{-}\}-dependent phosphorylation of NKCC date back to the 1990s (16, 43, 44), but no molecular mechanism has been proposed to date as how intracellular [Cl\(^{-}\)] is reduced in the face of hypertonic conditions. By measuring RVI after hypertonic cell shrinkage, we found that cell volume rapidly shrank by 30% under hypertonic conditions in both LZ and LRRC8A-KO cells (Fig. 3A). Cell shrinkage was followed by a slow increase in volume toward initial values, quantified as the cell volume recovered from the maximum shrinkage (% RVI). LZ cells reached ~40% RVI (Fig. 3B). In contrast, KO (Fig. 3B and C) and p38\(^{\text{KO}}\) cells (Fig. 3C) showed a marked reduction in RVI, similar to cells treated with DCPIB or with the inhibitors of p38 or MSK1 (Fig. 3D). The different RVI response between LZ and KO cells cannot be attributed to differences in the basal cell volume (SI Appendix, Fig. S5 A and B) or initial hypertonicity-induced shrinkage (SI Appendix, Fig. S5B) as both parameters did not differ between LZ and KO cells. RVI was also impaired in KD cells overexpressing the siRNA-resistant LRRC8A-S217A mutant, in contrast to cells overexpressing LRRC8A-WT, which showed a significant increase in RVI (Fig. 3E).

The NKCC inhibitor bumetanide (45) prevented RVI in LZ cells but not in KO cells (Fig. 3F), showing that the final effector responsible for increased RVI downstream of LRRC8A was the NKCC triple cotransporter. The involvement of LRRC8A in the activation of NKCC was also evaluated by measuring the halide-dependent quenching of YFP tagged to the intracellular side of NKCC1 (46). HeLa cells transfected with YFP-tagged NKCC1

LRRC8A Is Regulated upon Hypertonicity by the p38/MSK1 Kinase Pathway. We next investigated how the chloride channel activity was induced under hypertonic conditions. p38 SAPK is known to be activated by hyperosmolarity (38) and to mediate cell adaptation in hypertonic conditions (2, 38). We observed p38 phosphorylation (indicative of its activation) after a hypertonic challenge in both LZ and KO cells (SI Appendix, Fig. S4A). Notably, activity of LRRC8A-containing channels under hypertonic conditions was reduced by the p38 inhibitor SB203580 (39) (Fig. 2A) and in p38\(^{\text{KO}}\) cells (SI Appendix, Fig. S4B), consistent with a channel gating mechanism involving the p38 pathway. Genetic inhibition of p38 or MSK1 almost completely abolished MSK1-mediated quenching of YFP tagged to the intracellular side of LRRC8A (SI Appendix, Fig. S4C).

LRRC8A was phosphorylated under hypertonic conditions but not in HeLa-p38\(^{\text{KO}}\) cells (SI Appendix, Fig. S4D). However, no phosphorylation sites sensitive to the p38 inhibitor SB203580 were detected in residues of LRRC8A that are exposed intracellularly. Therefore, we focused on the mitogen- and stress-activated kinase 1 (MSK1), a downstream kinase directly regulated by p38 (40). Exposure to hyperosmotic shocks likewise triggered MSK1 phosphorylation (SI Appendix, Fig. S4E). MSK1 is primarily a nuclear kinase that also shuttles to the cytosol upon hypertonic stress (SI Appendix, Fig. S4F). Strikingly, inhibition of MSK1 with SB747651A (41) prevented LRRC8A phosphorylation in response to hyperosmotic stress (Fig. 2B) and, similar to p38 inhibition, decreased the level of LRRC8A activity (Fig. 2A). Next, we focused on identifying the LRRC8A residue(s) required for MSK1-mediated phosphorylation and activation under hypertonic conditions. LRRC8A contains several putative MSK1 phosphorylation sites (Fig. 2C). The intracellular loop (ICL) of LRRC8A was strongly phosphorylated in vitro by MSK1, and the mutation of Serine 217 to Alanine (S217A) almost completely abolished MSK1 phosphorylation in this region (Fig. 2D) and in the full-length LRRC8A (Fig. 2E). We next measured chloride currents in HeLa cells in which LRRC8A expression was stably knocked down by small interfering RNA (shRNA) (KD) (29) and overexpressed LRRC8A-wild type (WT) or LRRC8A-S217A (shRNA resistant). Indeed, LRRC8A-WT but not LRRC8A-S217A—expressing cells generated Cl\(^{-}\) currents after dialysis in low IS solutions and exposure to hypertonic conditions (Fig. 2F). We then examined whether genetic activation of MSK1 leads to LRRC8A activation in the absence of any osmotic stress or change in IS. Expression of a constitutively active MSK1 (MSK1-T588A and T704D) (42) spontaneously activated a DCPIB-sensitive Cl\(^{-}\} current in KD cells overexpressing LRRC8A-WT but not in those cells overexpressing LRRC8A-S217A channels (Fig. 2G). Thus, we demonstrated that activation of the p38/MSK1 pathway under hyperosmotic conditions is sufficient to activate LRRC8A even without changes to the intracellular IS.

LRRC8A Facilitates the WK/NKCC Axis to Support RVI and Cell Survival. We hypothesized that MSK1-mediated activation of LRRC8A under hypertonic conditions provides a pathway for Cl\(^{-}\} efflux that contributes to the activation of NKCC, and therefore, we addressed how LRRC8A participates in the cell volume response to hypertonic stress. Following exposure to hypertonic stress, shrunk cells immediately react by activating a rapid uptake of electrolytes via NKCC and the osmotically obliged water, thereby regaining the initial cell volume (1). Reports on the need to reduce intracellular [Cl\(^{-}\)] to trigger NKCC activity (12, 15) and the Cl\(^{-}\}-dependent phosphorylation of NKCC date back to the 1990s (16, 43, 44), but no molecular mechanism has been proposed to date as how intracellular [Cl\(^{-}\)] is reduced in the face of hypertonic conditions. By measuring RVI after hypertonic cell shrinkage, we found that cell volume rapidly shrank by 30% under hypertonic conditions in both LZ and LRRC8A-KO cells (Fig. 3A). Cell shrinkage was followed by a slow increase in volume toward initial values, quantified as the cell volume recovered from the maximum shrinkage (% RVI). LZ cells reached ~40% RVI (Fig. 3B). In contrast, KO (Fig. 3B and C) and p38\(^{\text{KO}}\) cells (Fig. 3C) showed a marked reduction in RVI, similar to cells treated with DCPIB or with the inhibitors of p38 or MSK1 (Fig. 3D). The different RVI response between LZ and KO cells cannot be attributed to differences in the basal cell volume (SI Appendix, Fig. S5 A and B) or initial hypertonicity-induced shrinkage (SI Appendix, Fig. S5B) as both parameters did not differ between LZ and KO cells. RVI was also impaired in KD cells overexpressing the siRNA-resistant LRRC8A-S217A mutant, in contrast to cells overexpressing LRRC8A-WT, which showed a significant increase in RVI (Fig. 3E). The NKCC inhibitor bumetanide (45) prevented RVI in LZ cells but not in KO cells (Fig. 3F), showing that the final effector responsible for increased RVI downstream of LRRC8A was the NKCC triple cotransporter. The involvement of LRRC8A in the activation of NKCC was also evaluated by measuring the halide-dependent quenching of YFP tagged to the intracellular side of NKCC1 (46). HeLa cells transfected with YFP-tagged NKCC1...
showed a decrease in the intensity of the YFP signal when challenged with hypertonic solutions (due to Cl\textsuperscript{−} entry via NKCC). Quenching was prevented in the presence of DCPIB or the NKCC inhibitor azosemide (45) (SI Appendix, Fig. S6 A and B). Taken together, RVI results obtained from both cell models (KO and KD cells) indicate that LRRC8A activation downstream of p38/MSK1 provides a pathway for Cl\textsuperscript{−} efflux and signals the triple cotransporter to take up electrolytes, thereby ensuring efficient RVI. If this mechanistic model is correct, imposing reduced intracellular [Cl\textsuperscript{−}] should overcome the deficient functional coupling of the LRRC8A and NKCC1 transport systems in LRRC8A-KO cells and ensure efficient RVI. To test this, we exposed cells to a hypotonic shock (to increase their volume and to reduce intracellular ion concentrations) followed by a return to isotonic conditions.
conditions, at which point cells shrink as a result of increased osmolarity and the efflux of water. This maneuver has been classically used for the preactivation of NKCC to achieve an efficient RVI in cells that do not up-regulate cell volume when challenged with hypertonic conditions (1, 14). In response to this treatment, cells typically engaged first in RVD, followed by RVI on returning to isosmotic conditions (Fig. 3G).

KO cells presented reduced RVD because of impaired LRRC8A-mediated Cl\(^-\) efflux (see also SI Appendix, Fig. S2E) but similar RVI to that of LZ cells (Fig. 3H). Under these conditions of reduced intracellular [Cl\(^-\)] due to previous hypotonic cell swelling, NKCC activation was favored, and both LZ and KO cells attained equally efficient RVI, even in the absence of LRRC8A, suggesting that disparities in the intracellular levels of Cl\(^-\) between LZ and KO cells may underlie the differences in RVI.

To understand the link between LRRC8A and NKCC activities, we focused on WNK kinases. The ubiquitously expressed WNK1 is autophosphorylated and activated by a reduction in intracellular [Cl\(^-\)], triggering its downstream targets SPAK and OSR1 kinases, which ultimately phosphorylate and activate NKCC1 (17–20, 47). WNK1 showed a reduced activation (measured using an anti–phospho-WNK-S382) in KO and KD cells when compared to LZ cells exposed to hypertonic solutions (Fig. 4A and SI Appendix, Fig. S7A and B). However, WNK1 phosphorylation was not different between LZ and KO cells exposed to the isotonic-hypotonic-isotonic protocol shown in Fig. 3G (Fig. 4B); this is consistent with our data showing that experimental reduction of intracellular [Cl\(^-\)] by previous exposure to hypotonic solutions improved the RVI of KO cells to the level of LZ cells. These results also suggest that LRRC8A-driven RVI regulation occurs upstream of the activation of the
WNK1–NKCC1 axis. To further confirm the link between LRRC8A-containing Cl− channels and WNK1 activation, first, we overexpressed shRNA-resistant LRRC8A in KD cells and demonstrated that, unlike mock, overexpression of LRRC8A rescued WNK1 phosphorylation in response to hypertonic stimulation ([SI Appendix, Fig. S7 C and D]. Second, for LRRC8A-mediated efflux of Cl− to regulate WNK1 activation, the change in intracellular [Cl−] should occur at very early stages of the cell response to hypertonic conditions. We addressed this point by measuring intracellular [Cl−] in cells overexpressing the GFP-based ratiometric Cl− sensor ClopHensor (48) ([SI Appendix, Fig. S8 A and B]) or loaded with the Cl− sensitive fluorescent dye MQAE ([SI Appendix, Fig. S8C]). Following exposure to hypertonic solutions, both LZ and KO cells showed a reduction in the fluorescence signal (indicating increased [Cl−]) due to cell shrinkage. However, while LZ cells rapidly increased the fluorescence signal (because of reduction in intracellular [Cl−]), KO cells did not reduce intracellular [Cl−] and maintained a decreased signal, thereby supporting the role of LRRC8A-mediated channels in the modulation of WNK1 by affecting intracellular [Cl−]. The involvement of WNK1 in the regulation of RVI by LRRC8A-containing Cl− channels was also assessed by transfecting LZ and KO cells with inactive WNK1-S382A or kinases that are constitutively active (17, 18) by mutating the phosphorylatable Ser382 (WNK1-S382E) or making the kinase Cl− insensitive and increasing its autophosphorylation (WNK1-L369F/L371F) ([SI Appendix, Fig. S9]). WNK1-S382E or WNK1-L369F/L371F overexpression, but not WNK-S382A, rescued RVI in KO cells, whereas none affected RVI levels in LZ cells (Fig. 4C). Finally, we studied cell survival under hypertonic conditions in KO cells expressing inducible LRRC8A-WT (KO-WT) or LRRC8A-S217A (KO-iS217A) proteins to levels similar to those found in control HeLa cells ([SI Appendix, Fig. S10A]). Expression of iWT but not iS217A increased cell survival to levels comparable with LZ cells (Fig. 4D). Similarly, transfection of KO cells with shRNA-resistant LRRC8A-WT and LRRC8A-S217A decreased cell survival in increased survival of LRRC8A-WT– but not LRRC8A-S217A–expressing cells under hypertonic conditions ([SI Appendix, Fig. S10B]).

Discussion

Our work unexpectedly reveals that swelling-activated chloride channels containing the LRRC8A subunit are also critical for cell survival under hypertonic conditions. Based on a genome-wide CRISPR/Cas9 screen, KO cell models, cell imaging, electrophysiological, and pharmacological and molecular tools, we demonstrate that LRRC8A works as a bidirectional osmotic stress response element: as a Cl− efflux pathway that favors loss of electrolytes during RVD, and as a regulator of the Cl−-sensitive WNK–NKCC axis to activate gain of electrolytes during RVI. Our results also reinforce the recent view about the role of LRRC8A-containing channels in cell responses to stresses other than cell swelling (49) and confirm the link between the need for correct NKCC regulation and increased cell fitness in the face of hypertonic stress (11). We propose a mechanism (Fig. 4E) by which activation of the p38/MSK1 pathway phosphorylates and activates the LRRC8A chloride and promotes Cl− efflux, which in turn facilitates shifting the conformational equilibrium of WNK1 from the chloride-bound inhibitory state toward the less-hydrated, active state triggered by hypertonicity (27). Thus, the combination of both direct activation of WNK1 by hyperosmotic stress and reduction in Cl−-mediated autophosphorylation may represent a mechanism to ensure optimal WNK1 activation and guarantee NKCC1-mediated transport to promote RVI and cell survival in response to hypertonic environments. Numerous reports over the last 30 y have documented the activation of NKCC by cell shrinkage and/or reduction in intracellular [Cl−] (45),15,16 and postulated that decreases in intracellular [Cl−] from its elevated hypertonicity-induced set point may participate in the activation of NKCC (16). Therefore, activation of LRRC8A under hypertonic conditions may

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**Fig. 3.** The p38/MSK1 pathway and LRRC8A regulate NKCC-mediated RVI. (A) Time course of relative changes in cell volume of LZ and KO HeLa cells (normalized to isotonic conditions) before and after exposure to a 30% hypertonic medium. Mean ± SEM (n = 6). (B) Percentage of RVI, calculated as the percentage of volume recovered following the initial cell shrinkage at the different time points after the hypertonic stress. (C) Mean RVI (%) calculated at 60 min in LZ (n = 6), KO (n = 6), or p38K-O (n = 9) HeLa cells. (D) Mean RVI (%) calculated at 60 min in HeLa cells superfused with DMSO (n = 7), the LRRC8A channel inhibitor DCP18 (n = 6), the p38 inhibitor SB203580 (n = 6), or the M51K1 inhibitor SB747651A (n = 4). (E) Mean RVI (%) calculated at 60 min in KD (n = 4) HeLa cells overexpressing shRNA-resistant WT (n = 6) or S217A (n = 4) LRRC8A channels. (F) Mean RVI (%) calculated at 60 min after LZ or KO HeLa cells were exposed to DMSO or 50 μM bumetanide. (G) Relative changes in cell volume measured before and after superfusion of LZ or KO HeLa cells with a 30% hypotonic medium. (H) Mean RVI (%), calculated at 60 min after return to isotonic medium, as measured in KO cells (n = 6). P values were determined by two-tailed Student’s t test (H), a Bonferroni’s all pairwise comparison (F), or one-way ANOVA followed by post hoc Dunnett’s test versus control group (Left Bar) (all others).
prevent excessive increase in intracellular [Cl\(^-\)], maintaining a level that although elevated compared to isotonic conditions optimizes WNK1 activation by hypertonic stimuli. Indeed, our results showing that genetic inhibition of LRRC8A reduced WNK1 phosphorylation and efficient RVI (also impaired using LRRC8A channel inhibitors) support the hypothesis that hypertonicity-induced activation of the WNK–NKCC axis is modulated by a reduction in intracellular [Cl\(^-\)]. Further support to the relevance of intracellular Cl\(^-\) on WNK–NKCC axis activation under cell shrinkage conditions came from experiments in which intracellular Cl\(^-\) was previously reduced by exposing cells to a hypotonic shock (Fig. 3G and H) or by overexpressing a Cl\(^-\)-insensitive WNK1 (Fig. 4C and SI Appendix, Fig. S9). Under these conditions, no differences in WNK1 phosphorylation or RVI were observed between LZ and KO cells, thereby suggesting that the effect of LRRC8A-containing channels on the modulation of the WNK1–NKCC axis is through the modulation of intracellular Cl\(^-\) concentrations. Actually, direct activation of the purified kinase domain of WNK by osmolytes is promoted by reducing [Cl\(^-\)] (27), in concordance with our findings that LRRC8A-mediated Cl\(^-\) efflux prevents excessive accumulation of intracellular Cl\(^-\) and promotes WNK1–NKCC activation in intact cells.

Despite the numerous experimental approaches used for the demonstration of the involvement of LRRC8A in the cell response to hypertonicity, our study is not exempt of limitations. One is the technical difficulty to directly record channel activity in the transition from isotonic to hypertonic conditions with a recording pipette attached to the cell membrane. Also, we cannot completely rule out that channel activation in response to hypertonic stress is solely mediated by MSK1-mediated phosphorylation, without...
involving changes in IS or other possible modulators that are triggered in response to hypertonic stress. Nevertheless, our results expressing active MSK1 as well as LRRCA-S217A mutants pointed to MSK1-mediated phosphorylation being the main mechanism for channel activation in the absence of changes in intracellular IS. Related to this, classical activation of LRRCA-type chloride channels is known to depend on the presence of intracellular ATP and the state of cell swelling, SO-520 being more active with small changes in IS and less relevant with rapid and larger changes in IS. This is consistent with the involvement of an ATP-dependent phosphorylation event in the gating of LRRCA-containing chloride channels in the face of small changes in IS.

Our data also shows that LRRCA links two phosphorylation pathways (p38/MSK1 and WNK1) participating in the cell response to osmotic stress and invites a review of the role of LRRCA in different pathophysiological conditions involving WNK (53) or MSK1 signaling (54, 55).

Materials and Methods
Drugs, antibodies, oligonucleotides, and plasmids used are described in SI Appendix, Table S2.

Genetic Screening and Analysis. To identify genes that are essential for hypertonic stress survival, an unbiased loss-of-function genetic screening was performed in HeLa cells using the CRISPR-Cas9 system (28). Cells expressing a constitutive hCas9 were infected with one of two lentiviral gRNA libraries ([Toronto KnockOut Library] TKO-1 or TKO-2) (28). Cells were selected by adding puromycin (1.5 μg/mL) at 24-h postinfec­tion, and multiplicity of infection (MOI) was determined after 48 h (T0) by comparison with nonse­lected infected cells (TKO-1, MOI = 0.34; TKO-2, MOI = 0.5). At this point, 20 × 10⁶ cells were collected for subsequent genomic DNA (gDNA) extraction and sequencing to ensure good library representation (T0). The rest of the cells were split into three replicates (A, B, and C), each covering a 200-fold representation of each library (20 × 10⁶ cells). Cells were then split every 3 d, always keeping a 200-fold representation of each library. At T6, cells were selected and 24 h later were challenged with hypertonic stress (150 mM NaCl) or kept in regular medium. One day after, they were counted and frozen (20 × 10⁶ cells) for subsequent gDNA extraction, library preparation, and next generation sequencing, which were carried out as in ref. 28. Briefly, genomic DNA was extracted from cell pellets using the QiAamp Blood Maxi Kit (Qiagen). gRNA inserts were amplified via PCR using primers harboring Illumina TruSeq adapters with IS and IS barcodes, and the resulting libraries were sequenced on an Illumina HiSeq2500. Each read was completed with standard primers for dual indexing with Rapid Run V1 reagents. The first 20 cycles of sequencing were “dark cycles” or base additions without imaging. The actual 26-base pairs read began after the dark cycles and contained two index reads, reading the IS sequences first, followed by IS.

Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout (MageCK) (56) was used to evaluate the quality of screening data and to identify overlapping genes. MageCK is a software designed to analyze genome-wide CRISPR-Cas9 screenings, and it computes a per-gene fitness effect score by integrating information from all sgRNAs targeting a given gene. This so-called robust rank aggregation (RRA) score indicates the degree of selection of a gene in a screening. Correct establishment of the KO library is a proxy for good quality of the screening. An efficiently established library should be depleted of genes essential for cell survival. For this reason, day 8 control and 6-well treated cells were compared against day 0 libraries using MageCK. Genes were then ranked by RRA, and a gene set enrichment analysis (GSEA) (57) was performed against the entire Kyoto Encyclopedia of Genes and Genomes (KEGG) database (SI Appendix, Table S3). As expected, essential terms were enriched among the top depleted genes (false discovery rate [FDR] < 0.01) (including RNA polymerase, proteasome, spliceosome, cell cycle, and ribosome), thereby proving the correct establishment of our KO libraries. Next, day 8 control and NaCl-treated cells were compared using MageCK to identify genes whose KO causes defects in cell proliferation upon hyperosmotic stress (SI Appendix, Table S4). After multiple testing correction using the Benjamini–Hochberg procedure, LRRCA/B/SWELL1 emerged as the top scoring candidate (Fig. 18; ref. 1, FDR = 0.06).

Quality control of reads and mapping. Raw sequencing reads from CRISPR-Cas9 samples were assessed for quality using FASTQC (version v0.11.5) and subsequently aligned to the sgRNA sequences of the TKO (28) (available at http://tko.cbr.utoronto.ca/) using BowTie (v1.2.1.1; parameters used: –m 1 –v 2) (58). Read counts for each sgRNA were tabulated and used for downstream analyses.

Quality control of the screening and identification of fitness genes. Downstream analyses were performed using MageCK (v0.5.6) (https://sourceforge.net/p/mageck/wiki/Home/) (56). GSEA for quality control of the screening (Day 0 versus Day 8 comparison) was done with MageCK's “pathway” command, with default parameters, using KEGG pathways (v6.0) from the Molecular Signatures Database (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp) (59) as input. Candidate fitness genes for hyperosmotic stress were identified using MageCK's “test” command with default parameters (SI Appendix, Table S4). In both cases, the whole TKO library, comprising two sublibraries (base and SI Appendix), was used. Both sublibraries were normalized to the median ratio (MageCK's default), merged into a single library, and analyzed with MageCK's “test” command without further normalization (–nomethod mode).

Cell Lines. Cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco) containing 10% fetal calf serum (Sigma) supplemented with 1 mM sodium pyruvate and 100 U/mL penicillin (Gibco) and cultured in a 5% CO₂ humidified incubator at 37 C. HeLa cells (human, cervical epithelial, female) were purchased from the American Type Culture Collection and HeLa cells stably expressing Cas9, from ref. 28. HeLa-shLRRC8A cells (KD) were kindly provided by Dr. A. Patapoutian, Department of Neuroscience, The Scripps Research Institute, La Jolla, CA (29).

Control HeLa Cas9-LZ cells, LRRCA-KO (KO) cells, p38-KO cells, and HeLa Cells LRRCA-KO clone 6 cells expressing LRRCA-WT (KO-WT) or LRRCA-S217A (KO-S217A) under an inducible promoter were generated as described below. gRNA targeting LacZ or KOs from the TKO-1 library (see gRNAs sequences; SI Appendix, Table S2) were cloned into the pLCKO vector. To produce lentiviruses, guidelines of the “The RNAi Consortium” at the Broad Institute (https://portals.broadinstitute.org/gpp/public/resources/protocols) were followed. Briefly, 60 to 70% confluent 293T cells were transfected with pLCKO together with packing vectors (pMD2G and pSPAX2) and switched to inactivated high-serum (30%) medium. At 24- and 48-h posttransfection, supernatants were collected, pooled, spun, and filtered to eliminate residual cells. Cas9-expressing HeLa cells were then infected with 200 to 300 μL virus together with polybrene; at 24 h after infection, they were transfected with puromycin (1.5 μg/mL) for at least 48 h. LRRCA KO clones were obtained by single-cell sorting in 96-well plates on FACSaria Fusion (Becton Dickinson). After clonal expansion, clones were checked by Western blot for the presence of LRRCA protein. LRRCA KO clone 6 was used to generate cell lines expressing LRRCA-WT and LRRCA-S217A under an inducible promoter following the transfection protocol described by Kowarz and colleagues (60). Briefly, cells were seeded in 6-well plates and transfected the following day with Lipofectamine 3000 (Invitrogen) using pS100X transposase plasmid and pSBtet-8N constructs with the LRRCA WT and mutants described in this work. At 24-h posttransfection, cells were selected with up to 5,000 μg/mL G418 (Sigma) for up to 1 wk. Induction was determined using a range of doxycycline (Sigma) concentrations (from 10 to 250 ng/mL), and protein levels were analyzed from cell extracts by Western blot.

Cell Transfection. Cells were transfected with 1 μg/mL polyethylenimine (PEI, Polysciences, 29369) (PEI:DNA ratio of 5:1) diluted in 150 mM NaCl (for patch clamp studies of MSK1 activation of LRRCA, YFP quenching, and MSK1 imaging) or with Lipofectamine 3000 (Invitrogen) when transfected with WNk1 mutants, siLRRC8A, or siRNA-resistant LRRC8A-WT or LRRC8A-S217A in KD cells. A ratio of 1:1 Lipo:DNA diluted in Opti-MEM (Gibco) was used, following manufacturer’s instructions. All experiments were performed between 24- and 36-h posttransfection, except for patch-clamp experiments, which were carried out in KD cells expressing an siRNA-resistant channel, which were performed between 48- and 72-h posttransfection.

Generation of Constructs. MSK1 putative phosphorylation sites in LRRCA were mutated on the pIRE5-EFGP construct (gift from the Patapoutian laboratory; note that the LRRCA sequence has two siRNA resistance regions) using the Q5 site-directed mutagenesis kit (NEB), following the manufacturer’s instructions. NEBaseChanger software was used for oligonucleotide design (SI Appendix, Table S2). The ICL fragments (amino acids F144 to D258) from WT or mutated LRRCA pIRE5-EFGP plasmids were cloned into EcoRI/NotI sites of the pGEX-6P-1 plasmid for bacterial expression by PCR amplification. Full-length WT LRRCA or S217A mutant were cloned into EcoRI/NotI sites of pGEX-6P-1 plasmid by PCR amplification with a forward oligonucleotide containing the myc tag after the EcoRI restriction site. MSK1 was subcloned from MSK1-Biotin2DHA plasmid (a gift from R. Gomis, Institute for Research in Biomedicine [IBB], Barcelona) into the EcoRI

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site of pGEX-6P-1 plasmid for GST purification or PCR amplified and cloned into NotI/XcoI sites of the pETM11 plasmid for His-tag purification. Constructs with LRRCA under an inducible promoter were generated by PCR amplification from the corresponding pIREs-EGFP plasmids with a reverse oligonucleotide carrying the CRISPR resistance nucleotide changes against the guide SC GD2 and cloned into the SfiI site of the pSB-BN plasmid. The pEBG-FLAG-WNK1 L369F L371F construct was generated by substituting the Mrel-Bu3βI region from the pDNAs FR1/T0 FLAG WNK1 L369F L371F into the pEBG-FLAG-WNK1 Wt plasmid.

**Cell Viability Assays.** Cells were seeded into 6-well plates and then challenged with the indicated NaCl concentration on the following day. After 24 h, supernatants and cells were collected, pelleted, washed in phosphate-buffered saline (PBS), and then stained with 1 μg/ml PI (Sigma). PI staining was assessed by flow cytometry in FACSCalibur using CellQuest software (Becton Dickinson). LRRCA KO cells expressing WT or mutant LRRCA under an inducible promoter were seeded in 12-well plates with doxycycline and were challenged with the indicated NaCl concentration on the following day. After 24 h, supernatants and cells were collected, pelleted, washed with PBS, and stained with 1 μg/ml PI. PI staining was assessed by flow cytometry in Gallios (Beckman Coulter), and profiles were generated by using FlowJo software. Alternatively, after 24 to 48 h of stress, plates were washed with PBS, fixed, and stained with crystal violet solution for 30 min at room temperature (RT), rinsed with water, and air dried. Viable cells attached to the plate stained with crystal violet, making the intensity of the staining directly proportional to cell viability.

**Protein Purification.** Recombinant GST proteins were expressed in *Escherichia coli* BL21 cells grown at 37 °C to an optical density (wavelength of 600nm) (OD600) of 0.5 for FCL-LRRCA and MSK1 and of 0.8 for full-length LRRCA proteins. GST-tagged proteins were induced for 3 h by adding 1 mM IPTG and then washing the culture temperature to 25 °C. After induction, cells were collected by centrifugation and resuspended in 1/50 volume of STE 1× buffer (100 mM NaCl, 10 mM Tris·HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 5% Triton X-100 supplemented with 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM benzamidine, 200 μM leupeptin, and 200 μG/mL pepstatin). Cells were lysed by brief ice-cold sonication with high-speed centrifugation (RT), rinsed with water, and air dried. Viable cells attached to the plate stained with crystal violet, making the intensity of the staining directly proportional to cell viability.

**YFP and Chloride Imaging.** Cells grown in coverslips and transiently transfected with halide-sensitive YFP constructs (37) were washed thoroughly with isotonic solution containing 110 mM NaCl, 5 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 10 mM Hepes, pH 7.4. Osmolarity was adjusted to 310 mOsm/l with 80 μM mannitol. The hypotonic solution (220 mOsm/l) contained 85 mM NaCl and no mannitol, while the hypertonic solution (410 mOsm/l) contained 185 mM NaCl or NaI. For YFP-NKCC1 imaging experiments, hypertonic solutions were prepared by adding 100 mM of mannitol (410 mOsm/l) to an isotonic solution in order to avoid changes in ionic concentrations that could affect the sensor. Video microscopic measurements of YFP fluorescence were obtained using an Olympus IX70 inverted microscope with a 20× or 40× oil-immersion objective. The excitation light (488 nm) was supplied by a Polychrome IV monochromator ( Till Photonics) and directed toward the cells under study by a 505DR dichromatic mirror (Omega Optical). Fluorescence images were collected by a digital charge-coupled device camera (Hamamatsu Photometrics). Images of basal fluorescence levels in isotonic solution were computed every 5 s and recorded for 2 min, followed by exposure to either 30% hypo- or hypertonic solutions for 5 min or 10 min, respectively. After this, 80 μL 200 mM NaI was added to induce YFP quenching (adapted from ref. 23). Alternatively, instead of a NaI buffer, a hypertonic NaCl solution was substituted for an equivalent solution containing an equimolar concentration of NaI. The YFP signal was normalized to 1 (set at 100% of YFP fluorescence) at 5 s before NaI addition. The percentage of fluorescence decay after NaI addition was calculated as the YFP quenching response.

**Patch-Clamp Experiments.** Whole-cell recordings were obtained as previously described (50) using an Axon 200A amplifier (Axon Instruments). Currents were acquired at 33 KHz and filtered at 1 kHz. The pClamp8 software (Axon Instruments) was used for pulse generation, data acquisition, and subsequent analysis. LRRCA-like chloride currents were measured in cells clamped at −60 mV and pulsed for 400 ms from −100 mV to +100 mV in 50-mV steps every 30 s. iCl whole-cell currents were measured using pipettes (2 to 3 MΩ) filled with a solution containing 100 mM N-methyl-D-glucamine chloride (NMDGC), 1.2 mM MgCl2, 1 mM EGTA, 10 mM Hepes, 2 mM NaATP, and 0.5 mM NaGTP (pH 7.3 and 300 mOsm). To achieve a controlled intracellular lS of 0.8, 78.2 mM NaCl was used instead of NMDGC and adjusted osmolality to ~410 mOsm. The external solution contained NMDGC at 100 mM (for iso- and hypotonic conditions) or 185 mM (hypertonic conditions), 0.5 mM MgCl2, 5 mM KC1, 1.8 mM CaCl2, 5 mM glucose, and 10 mM Hepes, pH 7.4. Osmolarity was adjusted to 310 (isotonic), 220 (hypotonic), or 415 (hypertonic) mOsm/l with mannitol.
hypertonic solution for 10 min, and calibrated with 0 and 120 mM [Cl−] in the presence of the Cl−/OH− ionophore exchanger tributylthiocarbamate (10 μM) and the K+−/Na+ exchanger mincain (5 μM) (TBTN). Calibration of ClopH sensor was performed in vivo. Cells (n = 7) were monitored in isotonic solution for 2.5 min and then perfused with standard solutions at 0, 15, 30, 50, 80, 110, and 120 mM [Cl−] for 4 min in the presence of TBTN. All calibration solutions contained 120 mM [K+] 20 mM [Na+], 1 mM Mg gluconate, 1 mM Ca gluconate, 5 mM glucose and 10 mM Hepes, pH 7.3 and 300 mM NaCl. The desired [Cl−] in each standard solution was achieved by a combination of Na and K gluconate replacing 10% nonfat dried milk powder. For the total-WNK1 condition, IPs were divided and loaded into two separate wells in the same gel to obtain both total- and phospho-WNK1 bands and thus the ratio of the two signals (phospho/total). For the phospho-WNK1 condition, the ratio to total-WNK1 signal was calculated using the total-WNK1 band obtained from the inputs. Primary antibodies were incubated overnight at 4 °C and secondary antibodies for 1 h at RT. Bands were developed with ECL substrate Clarity using the ChemiDoc XR+ system and quantified with Quantity One Software (BioRad).

**Statistical Analysis.** All electrophysiological, imaging, cell volume, and biochemical data are presented as mean ± SEM. Statistical analyses were performed using SigmaPlot software. First, a normality test was run, and for the data that followed normal distributions, a Student’s paired or unpaired t test was applied between two groups and one-way ANOVA followed by Bonferroni or Dunnnett post hoc tests across multiple groups. In the case of multiple groups, comparison of different conditions to a control group is run using the Dunnett’s method, whereas all pairwise comparisons were evaluated using Bonferroni’s method or one-way ANOVA followed by Holm– Sidak post hoc test, as suggested by the statistical package. For the data that did not assume Gaussian distributions, a Mann–Whitney U test was used for comparing two groups. The criterion for statistically significant difference was P < 0.05. Statistical analyses of data generated with the genetic screening were performed using bash and R scripts; visualizations were done using the ggplot2 (v3.1.0) (63) and ggrepel (v0.8.0) (64) R packages.

**Data Availability.** Gene data generated in this study have been uploaded to Gene Expression Omnibus under accession number GSE185316. The code generated for this study is available at GitHub (https://github.com/CellSignalingSWG11_Lelia_CSRIP screening_2020).

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**Conflict of Interest:** None declared.

**Data and Resources.** Gene expression data from gene expression screening were obtained using the GSE185316/GSE145496 (29). The code generated for this study is available at GitHub (https://github.com/CellSignalingSWG11_Lelia_CSRIP screening_2020).

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**Author Contributions.** E.K.H., I.H.L., S.F.F., and E.d.N. designed the experiments. E.K.H., I.H.L., S.F.F., and E.d.N. analyzed the data and wrote the manuscript. S.A., A.L., F.P., and R.B. provided essential reagents and technical expertise. E.K.H. and E.d.N. wrote the grant proposals. G.S., M.A.V., and C.R. provided essential reagents and technical expertise. E.K.H. and I.H.L. prepared the figures.

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