Correction

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Correction for “Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles,” by Qiang Ding, Brigitte Heller, Juan M. V. Capuccino, Bokai Song, Ila Nimgaonkar, Gabriela Hrebikova, Jorge E. Contreras, and Alexander Ploss, which appeared in issue 5, January 31, 2017, of Proc Natl Acad Sci USA (114:1147–1152; first published January 17, 2017; 10.1073/pnas.1614955114).

The authors wish to note the following: “It has been brought to our attention that the original Fig. 5A in our paper contained a duplicate image in which the panel showing the localization of ORF3 86-88A mutant was duplicated and also shown as ORF3 95-97A mutant. In the corrected version of Fig. 5A shown below, we have replaced the duplicate image with the correct original data. After careful reexamination of the original data, we are confident that the error in assembling this figure does not affect the validity of the research or the conclusions that we have drawn. We are grateful to the careful reader who brought this mistake to our attention and apologize for any confusion that this error may have caused.” The corrected Fig. 5 and its legend appear below.

Fig. 5. Identification of amino acid residues critical for HEV ORF3’s ion channel activity. (A) HepG2C3A cells lentivirally transduced with HA-tagged wild type or the indicated ORF3 mutant were stained with anti-HA and anti-calnexin antibodies. Nuclei were stained with Hoechst dye. Shown are representative images of triplicate experiments. (B) Current–voltage relationship of X. laevis oocytes expressing HEV wild type or the indicated ORF3 mutants. Experiments were conducted as detailed in Fig. 3. Data represent the mean ± SD (n = 5).

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Hepatitis E virus ORF3 is a functional ion channel required for release of infectious particles

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Hepatitis E virus (HEV) is the leading cause of enterically transmitted viral hepatitis globally. Of HEV’s three ORFs, the function of ORF3 has remained elusive. Here, we demonstrate that via homophilic interactions ORF3 forms multimeric complexes associated with intracellular endoplasmic reticulum (ER)-derived membranes. HEV ORF3 shares several structural features with class I viroporins, and the function of HEV ORF3 can be maintained by replacing it with the well-characterized viroporin influenza A virus (IAV) matrix-2 protein. ORF3’s ion channel function is further evidenced by its ability to mediate ionic currents when expressed in Xenopus laevis oocytes. Furthermore, we identified several positions in ORF3 critical for its formation of multimeric complexes, ion channel activity, and, ultimately, release of infectious particles. Collectively, our data demonstrate a previously undescribed function of HEV ORF3 as a viroporin, which may serve as an attractive target in developing direct-acting antivirals.

In this study, we aimed to further elucidate the function of ORF3. Building on a previously established cell culture system (17), we developed a transcomplementation system to uncouple HEV RNA replication from the assembly and release of infectious virions. This platform enabled us to discover and characterize a previously undescribed function of ORF3 as a viroporin—a virally encoded ion channel. Because HEV ORF3 shares multiple features with class I A viroporins, we tested whether its function could be substituted by another well-characterized viroporin, influenza A virus (IAV) matrix-2 (M2). Expression of IAV M2 can indeed partially rescue release of infectious virions. Furthermore, voltage-clamp experiments directly demonstrate that expression of HEV ORF3 can facilitate the flux of ions across the plasma membrane. Implication of HEV ORF3 is an ion channel.

Results

ORF3 Is a Transmembrane Protein Localized at ER Membranes and Forms Multimeric Complexes. Our initial bioinformatic analysis indicated that HEV ORF3 contained a putative transmembrane domain (Fig. L4). This prediction was corroborated following expression of HEV ORF3. ORF3 deletion results in 70,000 deaths and 3,000 stillbirths every year. Whereas most HEV infections occur in developing countries, recent epidemiological studies have found a high seroprevalence of anti-HEV antibodies in industrialized countries (1), suggesting exposure to the virus from travel to HEV endemic areas or from contact with pigs, a major reservoir of HEV. In a majority of cases, HEV causes an acute infection, but among immunocompromised patients—notably organ transplant recipients (2, 3) and individuals coinfected with HIV (4–6)—HEV can progress to chronicity. An effective vaccine preventing HEV infection has been developed, but it is only licensed in China (7). Pegylated IFN (peg-IFN) and the nucleoside analog ribavirin (8) have been used to treat HEV infection (8, 9), but the use of these drugs is not recommended in certain patient groups, including pregnant women and organ transplant recipients. Therefore, novel antiviral compounds are still needed, especially because HEV isolates resistant to RBV have been identified (10).

HEV is a quasi-enveloped, positive-sense RNA virus with three ORFs. HEV’s three gene products and their associated functions could serve as druggable targets. However, only ORF1 and ORF2 are the most fully characterized. ORF1 encodes for a nonstructural polyprotein composed of a methyltransferase, papain-like cysteine protease, RNA helicase, and RNA-dependent RNA polymerase (11). ORF2 encodes for the viral capsid protein and is involved in virion assembly, interaction with the host cell, and immunogenicity. It contains three glycosylation sites necessary for formation of infectious particles (12). In contrast, ORF3 and its corresponding function(s) have been largely elusive. The smallest ORF of the HEV genome, ORF3 is translated from a subgenomic RNA into a protein of 113–115 amino acids. Previous studies showed that ORF3 is bound to viral particles found in patient sera (13) and produced in cell culture (13, 14). Although in cultured cells ORF3 has not appeared essential for HEV RNA replication, viral assembly, or infection, it is required for particle release (14–16).

In this study, we aimed to further elucidate the function of ORF3. Building on a previously established cell culture system (17), we developed a transcomplementation system to uncouple HEV RNA replication from the assembly and release of infectious virions. This platform enabled us to discover and characterize a previously undescribed function of ORF3 as a viroporin—a virally encoded ion channel. Because HEV ORF3 shares multiple features with class I viroporins, we tested whether its function could be substituted by another well-characterized viroporin, influenza A virus (IAV) matrix-2 (M2). Expression of IAV M2 can indeed partially rescue release of infectious virions. Furthermore, voltage-clamp experiments directly demonstrate that expression of HEV ORF3 can facilitate the flux of ions across the plasma membrane.

Significance

Hepatitis E virus (HEV) is responsible for an estimated 20 million enterically transmitted cases of viral hepatitis globally. Here, we demonstrate that one of HEV’s three major gene products, ORF3, is an ion channel. Deletion of ORF3 abrogates release of infectious virions, and we show that viral egress can be rescued by expressing the influenza A virus (IAV) matrix-2 protein in trans. Expression of ORF3 facilitates ion flux across the plasma membrane, providing direct evidence for its viroporin activity. We identify regions within ORF3 abrogating both ion channel activity and particle release, thereby linking these two processes for a quasi-enveloped human virus and providing an attractive potential target for antiviral drug development.

Author contributions: Q.D., J.E.C., and A.P. designed research; Q.D., B.H., J.M.V.C., B.S., I.N., G.H., J.E.C., and A.P. performed research; Q.D., J.E.C., and A.P. analyzed data; and Q.D. and A.P. wrote the paper.

Conflict of interest statement: Q.D. and A.P. have filed a patent application on the use of the HEV transcomplementation system and ORF3’s ion channel function for anti-HEV drug screening.

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HEV ORF3 in HepG2C3A cells, a commonly used human hepatoma cell line permissive to HEV infection. In these cells, we observed colocalization of ORF3 with the ER-associated protein calnexin (Fig. 1B), suggesting ORF3 associates with intracellular membranes presumably derived from the ER. The punctate pattern in which ORF3 was expressed motivated us to determine whether the protein forms larger complexes. FLAG-tagged ORF3 immunoprecipitated with an anti-HA antibody in lysates derived from HepG2C3A cells coexpressing FLAG- and HA-tagged ORF3 (Fig. 1C). In contrast, coexpression of HA-tagged STING, a well-characterized ER-resident protein (19), did not pull down ORF3, demonstrating that the interactions between different ORF3 molecules are specific. Additionally, larger protein complexes were observed in Western blots of HepG2C3A lysates even under denaturing conditions, suggesting multimerization (Fig. 1D). Collectively, these data confirm that HEV ORF3 is a transmembrane protein localized at ER membranes (20) and forms multimeric complexes, presumably through homophilic interactions.

HEV ORF3 Resembles a Class IA Viroporin. When we examined the characteristics of ORF3 more closely, we noted its similarity to known viroporins, i.e., virally encoded protein complexes that serve as functional ion channels. Like other viroporins, ORF3 is a small hydrophobic protein that tends to oligomerize in ER-derived membranes. Similar to class IA viroporins such as IAV M2 (21), HIV-1 Vpu (22), or the coronavirus E protein (23), ORF3 has a short tail at the N terminus that resides in the ER lumen and a long cytosolic tail at the C terminus that is prone to phosphorylation at a serine in position 70 (24, 25). To enable direct analysis of ORF3's function, we expressed ORF2 and/or ORF3 lentivirally in HepG2C3A cells (Fig. 2A and B and Fig. 1A). These cells were subsequently transfected with in vitro transcribed RNA from a recombinant HEV genome derived from the KernowC1/p6 genome (17) in which ORF2 and ORF3 are replaced by a secreted version of Gaussia luciferase (Gluc), termed rHEVΔORF2/3[Gluc] (Fig. 2C). Supernatants collected from these cultures at day 5 post-transfection were then used to infect naïve HepG2C3A cells. The recombinant HEV subgenome replicated equally efficiently in all cells, irrespective of ORF2/3 expression. In cells expressing ORF2 only, infectious virions assembled but were retained intracellularly (Fig. 2D). Only supernatants collected from HepG2C3A cells expressing HEV ORF2/3 together, and not separately, led to robust reinfecction, as indicated by an ~35- to 45-fold increase in luciferase activity over background (Fig. 2C). Consistent with these luciferase data HEV RNA was also only significantly increased in HepG2C3A cells that had been exposed to supernatants from rHEVΔORF2/3[Gluc] transfected ORF2/3 producer cells (Fig. S1B). These data are consistent with previous reports showing that ORF2 is essential for packaging and ORF3 for release of infectious particles (16).

Next, we aimed to determine whether ORF3's essential function in HEV release could be replaced by IAV M2, a well-characterized class IA viroporin (21). Notably, infection of HepG2C3A cells with supernatants from HepG2C3A cells in which transfection of rHEVΔORF2/3[Gluc] was complemented with wild-type ORF3, wild-type M2, or M2 mutant proteins were studied by holding the membrane voltage of the oocytes at ~60 mV and then applying voltage step pulses every 10 mV from ~90 to ~60 mV. Both hyperpolarization and depolarizing pulses induced large currents with minimal time dependence that increased to a steady value immediately after the voltage pulse was applied (Fig. S3).
current was significantly larger than the endogenous current evoked by identical changes of membrane voltage in control oocytes injected with M2 mutant (A30P) mRNA or mock injected but was very similar to that observed in oocytes injected with wild-type M2 mRNA (Fig. 3C). Whole-cell instantaneous currents were examined under different ionic conditions to estimate the selectivity properties of the HEV ORF3 channel. Little difference in the reversal potential for each of the tested solutions suggested that HEV ORF3 formed nonselective ion channels (Fig. 3D and Fig. S4). Likewise, and in contrast to M2 channels that are selective to protons and display a shift in the reversal potential at acidic (5) and basic (10) pH, ORF3’s do not show significant changes in the reversal potential as expected for nonselective channels (Fig. 3 E and F). Together, these data demonstrate that HEV ORF3 serves as a functional ion channel but does not have selectivity for specific ions.

Identification of Domains Within ORF3, Which Are Required for Viroporin Function and Particle Release. Next, we aimed to systematically identify regions within HEV ORF3, important for its ion channel function and release of infectious HEV particles. Thus, we performed alanine scanning mutagenesis to change triplets of amino acids to alanine across the entire ORF3 protein. Lentiviral vectors expressing wild-type ORF2 and ORF3 (Fig. 4, Top), ORF3expressing naive HepG2C3A cells. Dually transduced cells were subsequently transfected with rHEVα/ORF2/3[Gluc] RNA and supernatants collected 5 d thereafter. To assess whether any of the ORF3 mutants affected release of HEV, supernatants were used to infect naive HepG2C3A cells. Several positions led to >90% reduction in particle release compared with HEV released from cells expressing wild-type ORF2 and ORF3 (Fig. 4, Top). In particular, we confirmed that ORF3 residues 86–89 and 95–98, each containing a PXXP motif previously shown to be necessary for HEV release (14, 15, 18, 27), serve essential functions. In addition, mutations in positions 11–13, 29–40, 59–61, 71–73, and 80–85 reduced virion release by 80–95%. Gluc levels in the transfected cells were equivalent across all experimental conditions, demonstrating that the observed differences cannot be simply attributed to differences in RNA transfection and/or HEV replication efficiency in the different producer cells (Fig. 4, Bottom). Next, we eliminated from the analysis ORF3 mutants that significantly affected protein stability. Western blots of lysates from HepG2C3A cells expressing HA-tagged ORF3 showed that all mutants except ORF3[RA29-31AAA] and ORF3[VV32-34AAA] could readily be detected with an anti-HA antibody (Fig. S5A) and those that did express well, with the exception of ORF3[CCCI1-13AAA], still formed higher molecular weight complexes indicative of multimerized ORF3. All stable mutants were then subjected to voltage-clamp experiments. Notably, ORF3[CCCI1-13AAA] and ORF3[IF59-61AAA] exhibited a significant decrease in ion flux across the membrane compared with oocytes expressing wild-type ORF3 (Fig. 5B and Fig. S6A–C). ORF3[PLA86-88AAA] and ORF3[PA95-97AAA], which had alanine triplets disrupting the PXXP motifs critical for interactions with components of the ESCRT machinery, and thereby interfere with the release of infectious particles through the vacuolar protein secretion pathway (14–16, 18, 28), did not diminish ion channel activity. Of note, none of these mutants, including ORF3[CCCI1-13AAA] and ORF3[IF59-61AAA], displayed vastly different subcellular localization compared with wild-type ORF3 (Fig. 5A and Fig. S5 A and B). Thus, residues 11–13 and 59–61 likely fall in regions important for ORF3’s ion channel function.

Altogether, our data demonstrate that ORF3’s ion channel activity is important for particle release, which is an additional, distinct function to the previously described, essential interactions of HEV ORF3 with components of the ESCRT pathway.

Discussion

In the present study, we provide evidence that HEV ORF3 is a functional ion channel required for the release of infectious vi-
The HEV ORF3 protein displays ion channel activity. (5) was replaced by gluconate. (7) A one-way ANOVA confirmed difference between the different groups regardless of rimantadine, were collected 5 d posttransfection with rHEVΔORF2/3[Gluc] RNA. Naïve HEV G2C3A cells were incubated with these supernatants. After 12 h, cells were washed and Gaussia luciferase activity quantified in the cell culture supernatants 4 d later. Results are presented as percentage relative to the carrier-treated control. Data represent the mean ± SD (n = 5). (D) ORF3 has little to no ion selectivity. Shown is the current–voltage relationship of the peak tail currents of HEV ORF3 at different pHs. (3, 27) Experimental Procedures. Oocytes were clamped at −80 mV, followed by a 1-s depolarization to +20 mV. The voltage was then stepped to test voltages ranging from −80 to +50 mV in 10-mV increments. Error bars show SEM (n = 7). A one-way ANOVA confirmed difference between the different groups (F(3, 27) = 34.187, P < 0.005). Tukey’s honest significant difference (HSD) tests revealed significant differences between the M2 at pH 5 and M2 at pH 10 (P < 0.004) for HEV ORF3.

Fig. 3. The HEV ORF3 protein displays ion channel activity. (A) IAV M2 can substitute ORF3 function. Cell culture supernatants from naïve HepG2C3A, or HepG2C3A cells transfected with HEV ORF2 and ORF3, M2 or M2(A30P), were collected 5 d posttransfection with rHEVΔORF2/3[Gluc] RNA. Naïve HepG2C3A cells were incubated with these supernatants. After 12 h, cells were washed and Gaussia luciferase activity quantified in the cell culture supernatants 4 d later. Data represent the mean ± SD (n = 6–9). *P < 0.05, **P < 0.01, and ***P < 0.001. (B) Inhibition of IAV M2 function with rimantadine (Sigma-Aldrich) suppresses release of HEV particles. Cell supernatants from naïve HepG2C3A or HepG2C3A cells transfected with HEV ORF2 and IAV M2 in the presence of indicated concentrations of rimantadine, were collected 5 d posttransfection with rHEVΔORF2/3[Gluc] RNA. Naïve HepG2C3A cells were incubated with these supernatants. After 12 h, cells were washed and Gaussia luciferase activity quantified in the cell culture supernatants 4 d later. Results are presented as percentage relative to the carrier-treated control. Data represent the mean ± SD (n = 5). (C) Current–voltage relationship of X. laevis oocytes expressing HEV ORF3, IAV M2, IAV M2(A30P) or control oocytes. During the current recording, the oocytes were bathed in Ringer solution. The standard voltage-clamp protocol consisted of rectangular voltage step pulses from −90 to +60 mV in 10-mV increments applied from a holding voltage of −60 mV. Each point represents the steady-state current (average current between 4,000–5,000 ms) at the corresponding voltage step. Data represent the mean ± SD (n = 5). (D) ORF3 has little to no ion selectivity. Shown is the current–voltage relationship of the peak tail currents of HEV ORF3 at different pHs. To assess monovalent cation selectivity, K+, Cs+ or Li+ replaced Na+ at equimolar concentration. These substitutions did not alter the reversal potential of the HEV ORF3 currents. In addition, no significant change in reversal potential was observed when 20 mM CaCl2 was added to the solution or when Cl− was replaced by gluconate. (E) ORF3 channels, unlike M2, are nonselective to protons. Current–voltage relationship of the instantaneous current of HEV ORF3 (green) and M2 (blue). To assess for proton permeability oocytes expressing M2 or ORF3 channels were exposed to bath solutions at pH 10 and 5 (Experimental Procedures). Oocytes were clamped at −80 mV, followed by a 1-s depolarization to +20 mV. The voltage was then stepped to test voltages ranging from −80 to +50 mV in 10-mV increments. (F) Graph shows average reversal potential (Erev) for HEV ORF3 (green) and M2 (blue) channels. Error bars show SEM (n = 7). A one-way ANOVA confirmed difference between the different groups (F(3, 27) = 34.187, P < 0.005). Tukey’s honest significant difference (HSD) tests revealed significant differences between the M2 at pH 5 and M2 at pH 10 (P < 0.004) for HEV ORF3.

only three nonenveloped viruses [simian virus 40 (SV40), coxsackie B virus (CBV), and polio virus] (reviewed in refs. 29, 30). Viroporins play a central role in promoting viral pathogenesis. For both enveloped and nonenveloped viroporins, viroporin function can influence cell entry and genome replication but is most frequently linked to virus release. In line with our observations of HEV ORF3, viroporin-defective viruses are often impaired in their ability to properly assemble and release infectious particles (31–36). The mechanisms underlying viroporin-mediated viral release remain opaque. Cell lysis and the subsequent release of infectious virions can be triggered by increased membrane permeability as a result of viroporin accumulation (36). It has also been proposed that viroporin insertion in cellular membranes may disrupt the electrochemical gradient by facilitating ion fluxes across membranes, thus dissipating the membrane potential of internal vesicles or the plasma membrane and thus stimulating viral budding (30).

HEV is a particularly intriguing example of a virus apparently reliant on viroporin function for viral release. Although classically defined as a nonenveloped virus, recent work has shown it can be shed from infected cells as a quasienveloped virus (37) similar to hepatitis A virus (HAV) (38). HAV and presumably other nonenveloped viruses, such as HEV, hijack intracellular membranes to shield from neutralizing antibody epitopes contained in the viral capsid. Quasienvelopment is also thought to promote HAV spread within the liver but is mechanistically incompletely understood (39). For HAV, it was demonstrated that the biogenesis of the membranes surrounding virions is dependent on host proteins associated with the ESCRT pathway, specifically VPS4B and ALIX (38). HEV also relies on components of the ESCRT pathway for virus budding through the interaction of PXXP motifs contained within ORF3 with TSG101 (15, 18). However, the important role of ORF3 viroporin activity was not known and thus adds a critical piece to forming a more complete picture of HEV release. Our present study lays the foundation for also gaining a deeper understanding of the budding of nonenveloped and other quasienveloped viruses. Future studies will focus on elucidating the structural details of how the ORF3 ion channel may function and possibly regulate the release of infectious virions.
Because viroporins are essential for the release of a variety of viruses, they are attractive targets for antiviral therapy. For example, it was previously shown that amantadine and rimantadine can inhibit the ion channel activity of M2 (40) and thereby interfere with the uncoating of IAV. Likewise, long alkyl iminosugar derivatives block HCV p7 function and decrease the release of infectious virions (41–43), and amiloride derivatives impair the ion channel activity of HIV-1 Vpu and severe acute respiratory syndrome coronavirus (SARS-CoV) E (44, 45). Besides pegylated IFN and ribavirin, both of which have substantial side effects, there are currently no specific drugs approved for the treatment of hepatitis E that directly interfere with the viral life cycle. HEV ORF3’s function as a viroporin may serve as a potential “druggable” target and could pave the way for the discovery of therapeutics that block this ion channel and thus disrupt HEV infectivity. This would address the important need for developing direct-acting antivirals to combat HEV in particularly vulnerable populations, such as pregnant women and immunocompromised individuals, who cannot be easily treated by current drug regimens.

**Experimental Procedures**

Additional procedures are described in detail in SI Materials and Methods.

**Cell Lines and Animals.** HEK293T cells (ATCC) and HepG2C3A cells (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (vol/vol) FBS, 50 IU/mL penicillin and streptomycin, in a humidified 5% (vol/vol) CO2 incubator at 37 °C. All experiments involving oocytes derived from *X. laevis* were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Rutgers University.

**Coimmunoprecipitation and Western Blot Assay.** Cell lysates were prepared from 5 × 10⁶ cells in a 10-mM Tris buffer (pH 7.5) containing 0.1% Nonidet P-40 and 1 mM EDTA, along with a mixture of protease and phosphatase inhibitors. Lysates were then incubated with Flag (M2, Sigma-Aldrich) antibody (1 μg, at 4 °C for 6 h), after which the immune complexes were precipitated with protein A sepharose. These immunoprecipitates were resolved on a 12% (wt/vol) SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and then analyzed by Western blot using anti-Flag (M2, Sigma-Aldrich) or anti-HA (clone HA-7, Sigma-Aldrich) antibodies. Membranes were then washed three times with TBS-T for 15 min total. Membranes were incubated with goat anti-mouse DyLight800-conjugated antibody (Thermo Scientific) at 4 °C overnight. Blots were then analyzed using the Odyssey imaging system (LI-COR).

**Fig. 4.** Identification via alanine scanning mutagenesis of essential positions within HEV ORF3 for release of infectious virions. HEV ORF3 mutants generated by changing triplets of amino acids to alanines across the entire ORF3 protein were lentivirally delivered to HepG2C3A cells expressing ORF2. Dually transduced cells were subsequently transfected with rHEVΔORF2/3[Gluc] RNA. Five days posttransfection, supernatants were collected and used to infect naïve HepG2C3A cells. Gaussia luciferase activity was quantified in the producer cells (Bottom) and in the cell culture supernatant 4 d postinfection (Top). The putative transmembrane regions (TM) are underlined. The asterisk marks the serine residue of ORF3, which can be phosphorylated. The two boxes mark the PXXP motifs within ORF3. Shown are averages and SDs of triplicate measurements of three independent experiments.

**Fig. 5.** Identification of amino acid residues critical for HEV ORF3’s ion channel activity. (A) HepG2C3A cells lentivirally transduced with HA-tagged wild type or the indicated ORF3 mutant were stained with anti-HA and anti-calnexin antibodies. Nuclei were stained with Hoechst dye. Shown are representative images of triplicate experiments. (B) Current–voltage relationship of *X. laevis* oocytes expressing HEV wild type or the indicated ORF3 mutants. Experiments were conducted as detailed in Fig. 3. Data represent the mean ± SD (n = 5).
The ORF3 or M2 cDNA was cloned into pSP64 vector with restriction sites for HindII (5' end) and BamHIII (3' end) enzymes. The plasmid was linearized by EcoRI digestion and transcribed in vitro to synthesized mRNA using the mMESSAGE mMICHEANE HIGH-yield capped RNA transcription SP6 kit (Ambion). Healthy X. laevis oocytes in stage V to VI were injected with 20 ng of mRNA per oocyte and incubated at 16 °C in an ND-96 solution. Two-electrode voltage clamp (OC-725C, Warner Instruments) was used to record the currents at 48 h postinjection. The oocytes were first bathed in standard Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl2 and 5 mM HEPES, pH 7.4) at room temperature and impaled with microelectrodes filled with 3 M KCl. Currents were generated by applying a 5-s voltage step protocol from −80 to +60 mV in 10-mV increments with a holding voltage of −60 mV. As for the ion selectivity, oocytes were clamped at −80 mV, followed by a 5-s depolarization to +60 mV. The voltage was then stepped to test voltages ranging from −80 to +50 mV in 10-mV increments. This was performed by a step from grants from Princeton University and an Investigator in Pathogenesis Award by the Burroughs Wellcome Fund to (A.P.). Q.D. is supported by a postdoctoral fellowship from the New Jersey Commission on Cancer Research (DHFS16PP007).

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