Missense mutations in inositol 1,4,5-trisphosphate receptor type 3 result in leaky Ca\(^{2+}\) channels and activation of store-operated Ca\(^{2+}\) entry
Missense mutations in inositol 1,4,5-trisphosphate receptor type 3 result in leaky Ca\textsuperscript{2+} channels and activation of store-operated Ca\textsuperscript{2+} entry

Lara E. Terry, Vikas Arige, Julika Neumann, Amanda M. Wahl, Taylor R. Knebel, James W. Chaffer, Sundeep Malik, Adrian Liston, Stephanie Humblet-Baron, Geert Bultynck, and David I. Yule

SUMMARY
Mutations in all subtypes of the inositol 1,4,5-trisphosphate receptor Ca\textsuperscript{2+} release channel are associated with human diseases. In this report, we investigated the functionality of three neuropathy-associated missense mutations in IP\textsubscript{3}R3 (V615M, T1424M, and R2524C). The mutants only exhibited function when highly over-expressed compared to endogenous hIP\textsubscript{3}R3. All variants resulted in elevated basal cytosolic Ca\textsuperscript{2+} levels, decreased endoplasmic reticulum Ca\textsuperscript{2+} store content, and constitutive store-operated Ca\textsuperscript{2+} entry in the absence of any stimuli, consistent with a leaky IP\textsubscript{3}R channel pore. These variants differed in channel function; when stably over-expressed the R2524C mutant was essentially dead, V615M was poorly functional, and T1424M exhibited activity greater than that of the corresponding wild-type following threshold stimulation. These results demonstrate that a common feature of these mutations is decreased IP\textsubscript{3}R3 function. In addition, these mutations exhibit a novel phenotype manifested as a constitutively open channel, which inappropriately gates SOCE in the absence of stimulation.

INTRODUCTION
Mutations in the inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptors (IP\textsubscript{3}Rs) are increasingly associated with human disease. IP\textsubscript{3}Rs are calcium (Ca\textsuperscript{2+}) channels predominantly located on the endoplasmic reticulum (ER) membrane, that mediate Ca\textsuperscript{2+} release from the ER Ca\textsuperscript{2+} store upon binding of IP\textsubscript{3}. IP\textsubscript{3} is produced as the result of a signal transduction cascade initiated by agonist binding to G protein-coupled receptors or receptor tyrosine kinases at the plasma membrane (PM). Both receptor classes result in the activation of phospholipase C isozymes, which cleave phosphatidylinositol 4,5-bisphosphate into membrane-bound diacylglycerol and freely diffusible IP\textsubscript{3}. The spatial and temporal aspects of the resulting Ca\textsuperscript{2+} release into the cytosol allow for the interaction of Ca\textsuperscript{2+} with various effectors leading to a diverse set of cell functions including apoptosis, gene transcription, fertilization, proliferation, growth and development, metabolism, fluid secretion, and exocytosis. A secondary effect of the Ca\textsuperscript{2+} release is the depletion of the ER Ca\textsuperscript{2+} store, which when sensed by the Stromal Interaction Molecule (STIM) activates Ca\textsuperscript{2+} entry into the cytoplasm through the Orai channels resident in the PM—a process known as store-operated calcium entry (SOCE). In order to maintain homeostasis, excess Ca\textsuperscript{2+} is removed from the cytosol through extrusion from the cell via the plasma membrane Ca\textsuperscript{2+} ATPase (PMCA), through reuptake into the ER via the sarcoplasmic ER Ca\textsuperscript{2+} ATPase (SERCA) or sequestration by mitochondria through the mitochondrial Ca\textsuperscript{2+} uniporter (MCU).

Three IP\textsubscript{3}R genes in the human genome (ITPR1, ITPR2, ITPR3) produce monomeric (~300 kDa) isoforms of the receptor (IP\textsubscript{3}R1, IP\textsubscript{3}R2, and IP\textsubscript{3}R3), which share ~60-70% sequence homology. A single subunit consists of a highly conserved N-terminal suppressor domain (SD; 1-226) and a ligand-binding domain (LBD; 227-578), which are based on previously identified domains and structures in rat IP\textsubscript{3}R1 (NP_001007236) and a highly conserved C-terminal channel pore and cytosolic tail (2201-2671). These termini are separated by a large, non-conserved regulatory and coupling domain (579-2200) where regulation by Ca\textsuperscript{2+}, ATP, PKA phosphorylation, and other small molecules and binding partners occurs. As a result, IP\textsubscript{3} binding to the N-terminal LBD, which is about...
70 Å from the channel pore induces long-range conformational changes in the cytosolic domain enabling pore opening.27,30

The three isoforms of the receptor are obligate tetramers and can assemble as both homo- or hetero-tetrameric complexes.33,34 While IP₃Rs are ubiquitously expressed, one isoform or a combination of isoforms are preferentially expressed in various tissues and cell types.35–39 Thus, the stoichiometry of the IP₃R tetramers is likely dependent upon the distribution and endogenous expression level of each isoform.30 Previous studies have established that IP₃R1 is highly expressed in the nervous system and is essential for the normal development of the nervous system. Mice engineered to lack native IP₃R1 often die prior to birth, or if born, suffer from severe ataxia and seizures prior to early postnatal death.41–43 In contrast, IP₃R2 and IP₃R3 are commonly co-expressed in the periphery such as in secretory cells of the digestive system and exocrine system though, they can also be expressed in isolation, for example, IP₃R2 in the sweat glands. As a result, IP₃R2 or IP₃R3 single knock-out mice develop subtle phenotypes.44,45 However, IP₃R2 and IP₃R3 compound knockout mice exhibit severe salivary and pancreatic insufficiencies resulting in death shortly after weaning due to the inability to assimilate macronutrients from dry food.44

Mutations in all three IP₃R isoforms, located in all the major functional domains, are associated with several human diseases.1,2 The majority of the mutations described thus far, are found in the IP₃R1 isoform and mainly impact nervous system function consistent with the IP₃R1 null mouse models.46–50 In contrast, fewer mutations have been identified in the IP₃R2 and IP₃R3 isoforms and are associated with diseases including Sézary Syndrome, familial isolated primary hyperparathyroidism (FIHP),51 anhidrosis,52 head and neck squamous cell carcinoma,53 neuropathy,54–60 and immunopathy.61 Since most reports present case studies, the functional consequences of particular mutations on the overall structure and function of the receptors remain poorly understood. Of those published, most previous studies indicate mutations in IP₃Rs are associated with either diminished or complete loss of channel activity,2,80,81 while a few mutations are associated with increased channel activity.82,83

Recently, there has been an increase in the number of IP₃R3 missense mutations reported (Figure 1). Most of these mutations are in the regulatory and coupling domain and associated with peripheral neuropathies.77–80 Two studies investigated the functional consequences of these mutations on Ca²⁺ signaling in patient-derived cells; however, the consequences of these mutations on IP₃R3 function were difficult to discern likely due to a contribution and compensation from endogenous wild-type IP₃R1 and IP₃R2 isoforms present in these cells.77,81 To unambiguously probe any alteration in human IP₃R3 function, in this study we generated individual stable cell lines expressing human IP₃R3 (hIP₃R3) V615M, T1424M, R2524C mutations associated with neuropathy in a HEK-3KO background. This cell line was previously generated in our laboratory by CRISPR-Cas technology to lack all the three native IP₃Rs.84 Interestingly, the hR V615M, T1424M, and R2524C stable cells lines all exhibited elevated basal intracellular Ca²⁺ concentration ([Ca²⁺]), reduced ER Ca²⁺ content and constitutive SOCE activation under basal conditions. These data are consistent with a “leaky” channel phenotype. However, all three mutations exhibited drastically...
different Ca\(^{2+}\) release profiles in response to agonist stimulation. The V615M was poorly functional, R2524C was non-functional, and T1424M exhibited increased function at low agonist concentrations. Overall, we report the functional consequences of three neuropathy-associated IP\(_3\)R3 mutations including the first reports of IP\(_3\)R disease-associated mutations that result in leaky channels.

**RESULTS**

While the functional effects of disease-associated mutations in IP\(_3\)R1 and IP\(_3\)R2 have been previously investigated, little work has been done to characterize mutations in the IP\(_3\)R3 isoform. Several novel mutations in IP\(_3\)R3 (Figure 1) have been identified in the past decade with most associated with hereditary motor and sensory neuropathies (HMSN) such as Charcot-Marie-Tooth (CMT), which is characterized by progressive distal motor and sensory impairments (Table S1). In this study, we characterized the functional consequences of three mutations in hIP\(_3\)R3 located at different locations within the regulatory and coupling domain or channel domain, in order to gain mechanistic insight into how these mutations alter channel function and ultimately contribute to the disease phenotype.

**The V615M mutation results in a poorly functional and “leaky” channel**

Valine 615 is located in the regulatory and coupling domain of the IP\(_3\)R3 armadillo repeat domain (ARM) 1 (Figure 2A), distal to the LBD. The hydrophobic/non-polar Val615 residue is replaced by a larger, hydrophobic/non-polar, sulfur-containing Met (V615M). Individuals from three generations of a Finnish family express this variant on a single IP\(_3\)R3 allele (Table S1). The patients were diagnosed with demyelinating neuropathy (CMT) inherited in an autosomal dominant manner, and presented with progressive muscle weakness, wasting in the legs and sometimes hands, as well as intermittently reduced nerve conduction values (NCV). The Val615 residue, is not conserved among hIP\(_3\)R isoforms, but is evolutionarily well conserved between IP\(_3\)R3 in different species (Figure S1A). We mutated the codon encoding the Val615 residue in hIP\(_3\)R3 cDNA to Met and expressed the variant in isolation in HEK-3KO cells (hR3 V615M) (Figure 2B). The hIP\(_3\)R3 V615M mutation localized properly to the ER membrane (Figure S1B).

Our approach was to generate several stable cell lines expressing hR3 IP\(_3\)R3 homo-tetramers at varying levels (Figure 2C) allowing for comparison of channel function with wild-type (WT) cell lines of a similar expression level. For example, HEK293 IP\(_3\)R1/2 double knockout cells expressing endogenous levels of IP\(_3\)R3 were previously generated through CRISPR/Cas9 gene editing to only express endogenous hIP\(_3\)R3 (Endo. hR3), while high IP\(_3\)R3 expressing HEK-3KO cells were generated by stably over-expressing hIP\(_3\)R3 cDNA (Exo. hR3). In Fura2/AM loaded hR3 V615M cells, agonist-induced Ca\(^{2+}\) release was utilized as a measurement of channel function. Prior to any agonist addition, all three V615M mutant cell lines exhibited significantly increased basal 340/380 ratios when compared to WT hR3 expressing cells and HEK-3KOs, indicative of elevated basal [Ca\(^{2+}\)] (Figure 2D and 2E). The mean basal [Ca\(^{2+}\)] correlated with V615M IP\(_3\)R3 protein level, such that the lowest expressing hR3 V615M cell line (hR3 V615M #503), with the expression between that of the Endo. hR3 and Exo. hR3, exhibited only a small increase compared to the null and WT cell lines. In contrast, the hR3 V615M #524 cell line, with expression slightly greater than that of the Exo. hR3, and the V615M #506 cell line, with expression several-fold greater than the Exo. hR3 cell line exhibited a much larger increase in basal [Ca\(^{2+}\)]. In response to stimulation with increasing concentrations of carbacol (CCh), a muscarinic receptor agonist, which results in an increase in intracellular IP\(_3\), all three hR3 V615M cell lines exhibited a significant attenuation of the initial Ca\(^{2+}\) release (Figure 2F) and percentage of responding cells (Figure S1C) compared to the Endo. hR3 and Exo. hR3 cell lines. Of note, those hR3 V615M cells with larger basal [Ca\(^{2+}\)], were those that exhibited decreased function, while the small percentage of individual hR3 V615M cells with larger changes in Ca\(^{2+}\) release were those with lower basal [Ca\(^{2+}\)] (Figure S1D).

In population-based Ca\(^{2+}\) imaging assays performed on a FlexStation3 with micro-fluidics, all three hR3 V615M cells lines similarly showed significantly diminished Ca\(^{2+}\) release (Figure 2G) together with the area under the curve (AUC) (Figure S1E) in response to increasing [CCh]. Likewise, in Total Internal Reflection Microscopy (TIRFM) experiments, measuring the Ca\(^{2+}\) release from small clusters of IP\(_3\)R3, the highest expressing hR3 V615M cell line exhibited significantly decreased numbers of Ca\(^{2+}\) puff sites (Figure 2H and 2I) and Ca\(^{2+}\) puff per cell (Figure 2H and 2J) as compared to the Exo. hR3 cell line (Video S1; Video S2). These results further support the single-cell Ca\(^{2+}\) imaging data showing that the hR3 V615M Ca\(^{2+}\) channel is poorly functional.

Next, we performed experiments to investigate the mechanism underlying the raised basal [Ca\(^{2+}\)] in the V615M hIP\(_3\)R3 cells. In contrast to WT hR3 expressing cells or HEK-3KOs, removal of extracellular Ca\(^{2+}\) led to an increased basal [Ca\(^{2+}\)]. Finally, we investigated the effect of extracellular Ca\(^{2+}\) on the Ca\(^{2+}\) release profile following stimulation with CCh. To do this, we stimulated hR3 V615M cell lines expressing varying levels of IP\(_3\)R3 with CCh in the absence or presence of extracellular Ca\(^{2+}\). As expected, the basal [Ca\(^{2+}\)] was significantly increased in the absence of extracellular Ca\(^{2+}\) (Figure 2D and 2E). However, the Ca\(^{2+}\) release profile following stimulation with CCh was not significantly different between the two conditions, indicating that the Ca\(^{2+}\) release profile is not affected by extracellular Ca\(^{2+}\).
Figure 2. hR3 V615M is a poorly functional Ca\(^{2+}\) channel with elevated basal cytosolic [Ca\(^{2+}\)]

(A) Chimera (PDB: 6DR0) was used to visualize Val615 (yellow) in ARM1 of the regulatory and coupling domain (red) of IP3R3.

(B) Cell lines with varying expression of human IP3R3 harboring the V615M mutation (hR3 V615M) were generated in IP3R-null HEK-3KO cells and western blotted alongside WT cell lines, either endogenously expressed hIP3R3 (Endo. hR3) and exogenously expressed hIP3R3 (Exo. hR3).

(C) Quantification of expression of hIP3R3 expression with respect to GAPDH in HEK-3KO (blue), Endo. hR3 (purple), Exo. hR3 (green), and hR3 V615M (pink, orange, red) cell lines. Colored lines represent the mean of at least n = 3 experiments, and error bars represent SEM. Averages were normalized to that of the Endo. hR3 cell line.

(D) Representative traces of Ca\(^{2+}\) signals in fura-2 loaded cells in response to the addition of indicated concentrations of CCh.

(E) Scatterplot summarizing the basal Ca\(^{2+}\) (average of the initial 20,340/380 ratio points in Ca\(^{2+}\)-containing media) from experiments similar to those in (D).

(F) Scatterplot summarizing change in amplitude (Peak 340/380 ratio – Basal 340/380 ratio (E)) of cell lines in response to CCh in single-cell imaging experiments similar to those in (D).

(G) Dose-response curve showing the change in amplitude of Ca\(^{2+}\) signals of Fura-2/AM loaded cell lines when treated with increasing [CCh] using a FlexStation3 96-well plate reader.

(H) Representative traces of Ca\(^{2+}\) puffs recorded by TIRFM in Exo. hR3 or hR3 V615M. Uncaging of ci-IP3 at the arrowhead.

(I) Number of Ca\(^{2+}\) puff sites per cell in Exo. hR3 (green) and hR3 V615M (red) cell lines loaded with Cal-520 in TIRFM experiments and treated with 1 mM ci-IP3.

(J) Number of Ca\(^{2+}\) puff per cell in Exo. hR3 (green) and hR3 V615M (red) cell lines loaded with Cal-520 in TIRFM microscopy experiments and treated with 1 mM ci-IP3. All data are mean ± SEM of at least three (N = 3) independent experiments. ***p < 0.001 when compared to HEK-3KO; **p < 0.01 when compared to Endo. hR3; and ***redp < 0.001 when compared to other stably expressed hR3 V615M cell lines; one-way ANOVA with Tukey's test performed in E, F and two-tailed t-test performed in (I and J).
resulted in a significant decrease in basal cytosolic \([\text{Ca}^{2+}]_i\) in the hR3 V615M cell lines (Figures 3A and 3B) to levels similar to the IP3R null and WT cell lines. A direct correlation was observed between the basal \([\text{Ca}^{2+}]_i\) and the magnitude of change in amplitude seen following the removal of extracellular Ca\(^{2+}\) (Figure S2A).

These data indicate that Ca\(^{2+}\) influx across the PM contributed to the elevated basal \([\text{Ca}^{2+}]_i\) in hR3 V615M expressing cells. Thus, we hypothesized that the elevated cytosolic Ca\(^{2+}\) was derived, at least in part, from Ca\(^{2+}\) leaking from the ER store through the mutant channel and subsequently gating SOCE. First, to evaluate the ER Ca\(^{2+}\) store content, the SERCA pump was inhibited by exposure to 30 \(\mu\)M CPA allowing the measurement of the ER Ca\(^{2+}\) store content.

Depletion of the ER Ca\(^{2+}\) store triggers SOCE through the interaction of STIM and Orai proteins. To investigate the contribution of Ca\(^{2+}\) influx via Orai-based channels to the elevated [Ca\(^{2+}\)] in the hR3 V615M mutants, we utilized the Orai inhibitor GSK-7975a. In HEK293 cells, GSK-7975a inhibited 67.4%
of CCh-induced Ca\(^{2+}\) influx (Figure S3A and S3B) and 50.8% of CPA-induced Ca\(^{2+}\) influx (Figure S3C and S3D). The addition of GSK-7975a resulted in a significant decrease in the basal [Ca\(^{2+}\)], in the hR3 V615M cell lines, which was not observed in the WT hR3 and HEK-3KO cell lines (Figures 3D, 3E, and S3E). The elevated basal [Ca\(^{2+}\)] levels correlated with a greater effect of GSK-7975a to reduce the elevated basal [Ca\(^{2+}\)] levels (Figure 3F). In total, these results suggest that the mutation of V615M in hIP3R results in a poorly functional, “leaky” channel. Ca\(^{2+}\) leak through the mutant channel leads to the partial depletion of ER Ca\(^{2+}\) stores thereby activating SOCE via Orai-based channels.

**The T1424M mutation results in a “leaky” channel that is functional**

The T1424M variant was identified as one of the two potential candidate gene mutations in a mother and her two daughters diagnosed with late-onset lower limb muscle weakness and sensory loss (Table S1, Phenotypes of relevant IP3R mutations associated with neuropathy, related to Figure 1).\(^{79}\) In these subjects, the hydrophilic/polar Thr1424 harboring a hydroxyl group is exchanged for a larger, hydrophobic/non-polar Met (T1424M) containing a sulfur group. Again, this mutation is present only on one allele [ITPR3] and thus the effect of the mutation appears to be dominantly inherited. The T1424M mutant is located within the regulatory and coupling domain of IP3R in the armadillo repeat 2 domain (ARM2) (Figure 4A). The T1424 residue is conserved both among all IP3R isoforms, as well as across multiple species in hIP3R (Figure S4A). Multiple cell lines stably expressing hR3 T1424M in HEK-3KO were generated with the hR3 T1424M #203 cell line expressing a low level of the IP3R3 similar to that of the Endo. hR3, while the hR3 T1424M #209 and #204 cell lines expressed intermediate amounts of IP3R3 between that of the Endo. hR3 and Exo. hR3 (Figures 4B and 4C). Homo-tetramers of hR3 T1424M were properly localized to the ER membrane (Figure S4B).

While the low IP3R3 expressing hR3 T1424M line (#203) did not exhibit an increased basal [Ca\(^{2+}\)] (Figures 4D and 4E), the intermediate level expressing hR3 T1424M (#209) and hR3 T1424M (#204) cell lines both exhibited significantly increased basal [Ca\(^{2+}\)] (Figures 4D and 4E). Ca\(^{2+}\) release in the low expressing hR3 T1424M cell line was refractory in response to any tested (CCh). In contrast, the Endo. hR3 cell line, with the most similar expressions exhibited robust responses to [CCh] greater than 3 \(\mu M\) (Figures 4F and S4C). Notably, the higher expressing hR3 T1424M cell lines, both exhibited significant Ca\(^{2+}\) release and number of responding cells following stimulation with lower [CCh] (Figures 4F and S4C). Once again, individual hR3 T1424M cells with elevated basal [Ca\(^{2+}\)], exhibited smaller changes in CCh-induced Ca\(^{2+}\) release than cells with more normal basal [Ca\(^{2+}\)] (Figure S4D). Remarkably, despite only intermediate levels of IP3R3 expression in the hR3 T1424M #209 and #204 cell lines the amplitude of Ca\(^{2+}\) release was greater in these lines than the WT hR3 cell lines at lower [CCh] indicating that the mutant channel is perhaps in a pre-activated confirmation (Figure 4F). Nevertheless, at higher [CCh] greater Ca\(^{2+}\) release was observed in the WT hR3 lines reinforcing the importance of this conserved residue for proper gating of the channel. In population-based FlexStation3 assays, the functionality of the higher expressing hR3 T1424M cell lines was confirmed and exhibited a shift in sensitivity at lower [CCh] (Figures 4G and S4E).

Notably, in TIRFM imaging experiments, spontaneous Ca\(^{2+}\) puffs were readily evident in the hR3 T1424M #204 cell line but were uncommon in Exo. hR3 expressing cells (Figure 4H) indicating that the mutant channel is active, even under basal conditions in absence of agonist stimulation (Figure 4, Video S3, Video S4). Additionally, upon stimulation through the uncaging of ci-IP3, the number of Ca\(^{2+}\) puffs and Ca\(^{2+}\) puff sites per cell (Figure 4J) were significantly higher in the hR3 T1424M #204 cell line as compared to the Exo. hR3 cell line and signal tended to globalize in the hR3 T1424M expressing cells (Video S5 and Video S6).

Despite differences in functionality, the elevated basal [Ca\(^{2+}\)], of the higher expressing hR3 T1424M cell lines again suggests that the T1424M mutation results in a leaky IP3R3 similar to the V615M mutation. In the higher expressing hR3 T1424M cell removal of extracellular Ca\(^{2+}\) resulted in a significantly larger reduction in [Ca\(^{2+}\)], than in the HEK-3KO and WT hR3 cell lines (Figures S5A and S5B). Similarly, the magnitude of the elevated basal [Ca\(^{2+}\)], of individual hR3 T1424M mutant cells correlated with larger changes in the [Ca\(^{2+}\)], following the removal of extracellular Ca\(^{2+}\) (Figure S5A). SERCA pump inhibition resulted in a significantly smaller Ca\(^{2+}\) leak from the ER Ca\(^{2+}\) store in the hR3 T1424M mutant cell lines with elevated basal [Ca\(^{2+}\)], levels (Figures S5A, S5B, and S5C) and the degree of inhibition was again correlated with the magnitude of the elevated basal [Ca\(^{2+}\)] in individual cells (Figures S5C and S5D). Similar to V615M expressing cell lines, the Orai inhibitor GSK-7975a effectively decreased the elevated basal [Ca\(^{2+}\)] (Figures S5D-S5F and S5E) of the higher expressing hR3 T1424M mutant cell lines. Overall, these data demonstrate that expression of...
Figure 4. hR3 T1424M increased Ca^{2+} channel function with elevated basal cytosolic [Ca^{2+}]

(A) Chimera (PDB: 6DR0) was used to visualize Thr1424 (yellow) in the regulatory and coupling domain (red) of IP₃R3.

(B) Cell lines containing varying expression of human IP₃R3 harboring the T1424M mutation (hR3 T1424M) were generated in IP₃R-null HEK-3KO cells and western blotted alongside WT cell lines; Endo. hR3 and Exo. hR3.

(C) Quantification of hIP₃R expression with respect to GAPDH of HEK-3KO (blue), Endo. hR3 (purple), Exo. hR3 (green), and hR3 T1424M (pink, orange, red) cell lines. Colored lines represent the mean of at least n = 3 experiments, and error bars represent SEM. Averages were normalized to that of the Endo. hR3 cell line.

(D) Representative traces of Ca^{2+} signals in cells expressing the indicated variant in response to the addition of increasing [CCh].

(E) Scatterplot summarizing the basal Ca^{2+} (average of the initial 20,340/380 ratio points in Ca^{2+}-containing media) from experiments similar to those in (D).

(F) Scatterplot summarizing change in amplitude (Peak 340/380 ratio – Basal 340/380 ratio (E)) of cell lines in response to increasing [CCh] in single-cell imaging experiments similar to those in (D).

(G) Dose-response curve showing the change in amplitude of Ca^{2+} signals when treated with increasing [CCh] using a FlexStation3 96-well plate reader.

(H) Representative traces of Ca^{2+} puffs in the absence of stimulation in Exo. hR3 and hR3 T1424M expressing cells.

(I) Number of Ca^{2+} puff sites and Ca^{2+} puffs per cell in Exo. hR3 (green) and hR3 T1424M #204 (red) cell lines loaded with Cal-520 in TIRFM microscopy experiments without photo-release of ci-IP₃.

(J) Number of Ca^{2+} puff sites and Ca^{2+} puffs per cell in Exo. hR3 (green) and hR3 T1424M (red) cell lines loaded with Cal-520 in TIRFM microscopy experiments following photo-release of 0.1 μM ci-IP₃. All data are mean ± SEM of at least three (N = 3) independent experiments. Control HEK-3KO (blue), Endo. hR3 (purple), and Exo. hR3 (green) data in E, F repeated from Figure 2 above. ***p < 0.001, **p < 0.01, *p < 0.05 when compared to HEK-3KO; ***(red)p < 0.001, **(red)p < 0.01 when compared to other stably expressed hR3 T1424M cell lines; one-way ANOVA with Tukey’s test performed in (E and F) and two-tailed t-test performed in (I and J).
hR3 T1424M at levels greater than that of Endo. hR3 led to a leaky channel that results in the inappropriate 
activation of SOCE in the absence of stimulation similar to the hR3 V615M mutation. However, unlike the 
V615M mutation, which resulted in poorly functional IP3R3 channels, the T1424M mutation, when over-ex-
pressed, leads to a variant with increased channel function at threshold levels of stimulation when 
compared to WT hIP3R3 lines with comparable expression.

The R2524C mutation forms a functionally dead but leaky channel
The R2524 residue is located in the channel domain at the junction of the 6th transmembrane (TM) and the 
linker (LNK) domain (Figure 6A) and is highly conserved in hIP3R subtypes and across different species (Fig-
ure S6A). The R2524C mutation was reported as a de novo mutation in two unrelated individuals (Table S1, Phenotypes of relevant IP3R mutations associated with neuropathy, related to Figure 1). One individual, 
harboring solely the R2524C mutant on one ITPR3 allele, exhibited a phenotype associated with neuropa-
thy.77 The second individual, who also expressed an additional mutation (R1850Q) on their second 
ITPR3 allele, also presented with immunopathy symptoms resulting in a diagnosis of severe combined immuno-
deficiency disease (Table S1).80 In these individuals, the positively charged Arg2524 residue is mutated to 
an uncharged, smaller Cys with a reactive thiol (-SH) group. The R1850Q mutation is present in 5% of the 
population as a polymorphism and when present alone in a parent of this individual did not result in an

Figure 5. hR3 T1424M cell lines exhibited depleted ER [Ca2+] and SOCE in the absence of agonist stimulation
(A) Representative traces of changes in cytosolic [Ca2+] following removal of extracellular Ca2+ in HEK-3KO (blue), Endo. hR3 (purple), Exo. hR3 (green), and 
hR3 T1424M (pink, orange, red) cell line. Cells were subsequently treated with 30 μM CPA allowing the measurement of the ER Ca2+ store content.
(B) Scatterplot summarizing the change in the basal 340/380 ratio following removal of extracellular Ca2+ in experiments similar to those in (A). Colored lines 
represent the mean of at least n = 3 experiments, and error bars represent SEM.
(C) Scatterplot summarizing the correlation between an elevated basal 340/380 Ca2+ ratio (Figure 2E) and the change in 340/380 ratio following treatment 
with 30 μM CPA (maximum CPA-induced amplitude – basal 340/380 ratio following removal of extracellular Ca2+) in experiments similar to those in (A). 
(D) Representative traces of Ca2+ signals in response to the addition of 10 μM GSK-7975a when loaded with Fura-2/AM.
(E) Scatterplot summarizing the change in 340/380 ratio following the addition of GSK-7975a (average of 20,340/380 ratio points prior to GSK-7975a 
addition – average of 20,340/380 ratio points following 200 s of GSK-7975a addition) from experiments similar to those in (D).
(F) Scatterplot summarizing the correlation between an elevated basal 340/380 Ca2+ ratio (Figure 2E) and the change in 340/380 ratio following treatment 
with 10 μM GSK-7975a (Figure 2E) and the change in 340/380 ratio following treatment with 10 μM GSK-7975a in experiments similar to those in (D). All data are mean ± SEM of at least three (N = 3) independent experiments. Control 
HEK-3KO (blue), Endo. hR3 (purple), and Exo. hR3 (green) data in (B, C, and E), and F repeated from Figure 3 above. ***p < 0.001 when compared to 
HEK-3KO; **p < 0.01 when compared to Endo. hR3; ***p < 0.001 when compared to Exo. hR3, and ***redp < 0.001 when compared to other stably 
expressed hR3 T1424M cell lines; one-way ANOVA with Tukey’s test performed in (B and E).
obvious phenotype and was previously characterized as poorly functional only when expressed at endogenous levels.80 We therefore mutated the Arg2524 residue to Cys in WT hIP3R3 (hR3 R2524C) and stably expressed the mutant in IP3R-null HEK-3KO cells. As before, we generated several stable cell lines of hR3 R2524C homo-tetramers with varying expression levels (Figure 6B). The hR3 R2524C mutant was expressed at similar levels to Endo. hR3 in the hR3 R2524C #607 line (Figures 6B and C). hR3 R2524C #612 and #616 exhibited expression levels similar to and greater than Exo. hR3, respectively. The hR3 R2524C mutant appeared properly localized to ER membranes (Figure S6B). The basal [Ca2+]i levels of the hR3 R2524C mutant cell lines were significantly increased in the absence of stimulation and the extent of the increase correlated with the relative expression level of the mutant (Figures 6Da and 6E). However, in contrast to the V615M and T1424M mutants, channel function in the hR3 R2524C mutant in response to stimulation was completely absent (Figures 6Fa and S6C), irrespective of the basal [Ca2+]i (Figure S6D) when measured using single cell or population-based80 paradigms.

The removal of extracellular Ca2+ again resulted in a decrease in basal [Ca2+]. in the high R2524C expressing cells (Figures 7A, 7B, and S7A). Similarly, the CPA-induced Ca2+ leak from the ER was also decreased in the higher expressing hR3 R2524C cell lines indicating reduced ER Ca2+ store content in these cell lines (Figures 7A, 7C, and S7B-S7D). Addition of GSK-7975a resulted in a significant decrease in the elevated basal Ca2+ of the higher expressing hR3 R2524C cell lines in the absence of stimulation (Figures 7D-7F and S7E), again suggesting the inappropriate activation of SOCE. Overall, these results indicate that the R2524C mutant is a completely non-functional Ca2+ channel, which again exhibits a leaky phenotype similar to the V615M and T1424M mutants.
DISCUSSION

While disease-associated mutations have been identified in all three human isoforms of the IP$_3$R, the functional consequences of mutations in IP$_3$R3 are yet to be determined. Previous reports using patient-derived fibroblasts with neuropathy-associated IP$_3$R3 mutations exhibited drastically disparate mRNA levels and protein expression of all three IP$_3$R isoforms. While the patient's fibroblasts exhibited a trend toward decreased Ca$^{2+}$ signaling, the presence and potential compensation by endogenous IP$_3$R1 and IP$_3$R2 isoforms may have confounded elucidating the full, functional effect of these mutant IP$_3$R3. As a result, no consensus could be reached regarding any change in channel function observed in fibroblasts harboring the V615M or compound heterozygous R1850Q/R2524C mutation.

Our functional studies were designed to study the mutations as homotetramers on an unambiguously null background. We demonstrate that all mutations tested exhibited markedly reduced or absent function when expressed at levels close to endogenous hR3 levels in IP3R-null HEK-3KO cells.

Over-expression of hR3 V615M, T1424M, and R2524C led to an elevated basal [Ca$^{2+}$]$_i$, decreased ER store Ca$^{2+}$ content, and subsequent inappropriate activation of SOCE through ORAI channels. This phenotype is consistent with these mutations resulting in a constitutively leaky IP$_3$R3 channel pore, revealed as over-expression results in Ca$^{2+}$ leak through the receptor, effective coupling to the SOCE machinery, and subsequent Ca$^{2+}$ influx across the PM that is not matched by Ca$^{2+}$ clearance mechanisms resulting in an elevated basal [Ca$^{2+}$].

Figure 7. hR3 R2524C cell lines exhibited depleted ER [Ca$^{2+}$] and SOCE in the absence of agonist stimulation

(A) Representative traces of changes in cytosolic [Ca$^{2+}$] following removal of extracellular Ca$^{2+}$ in HEK-3KO (blue), Endo. hR3 (purple), Exo. hR3 (green), and hR3 R2524C (pink, orange, red) cell lines. Cells were subsequently treated with 30 µM CPA allowing the measurement of the ER Ca$^{2+}$ store content.

(B) Scatterplot summarizing the change in the basal 340/380 ratio following removal of extracellular Ca$^{2+}$ in experiments similar to those in (A). Colored lines represent the mean of at least n = 3 experiments, and error bars represent SEM.

(C) Scatterplot summarizing the correlation between an elevated basal 340/380 Ca$^{2+}$ ratio (Figure 2E) and the change in 340/380 ratio following treatment with 30 µM CPA (maximum CPA-induced amplitude – basal 340/380 ratio following removal of extracellular Ca$^{2+}$) in experiments similar to those in (A).

(D) Representative traces of Ca$^{2+}$ signals in the indicated cell lines in response to the addition of 10 µM GSK-7975a.

(E) Scatterplot summarizing the change in 340/380 ratio following the addition of GSK-7975a (average of 20,340/380 ratio points prior to GSK-7975a addition – average of 20,340/380 ratio points following 200 s of GSK-7975a addition) from experiments similar to those in (D).

(F) Scatterplot summarizing the correlation between an elevated basal 340/380 Ca$^{2+}$ ratio (Figure 2E) and the change in 340/380 ratio following treatment with 10 µM GSK-7975a (E) in experiments similar to those in (D).
combination with reduced IP₃-stimulated activity, it is possible that even when expressed at endogenous levels, that the leaky phenotype of these variants contributes to the etiology of the disease in affected individuals through local, inappropriate activation of effectors. In this scenario, a blocker of the IP₃R₃ pore may ameliorate disease, if other IP₃R subtypes present can compensate for the decreased mutant IP₃R₃ channel activity.

Over-expression also revealed that hR3 V615M and T1424M retained, to some limited extent, their ability to be gated by elevated IP₃, while hR3 R2524C was refractory to stimulation and thus appears to form a dead channel. Only following expression at levels similar to, or greater than that of Exo. hR3 was channel function measurable. We have previously shown that increased expression of IP₃R mutants in HEK-3KO cells compared to IP₃R-null, chicken lymphocyte DT40-3KO cells facilitated measurable changes in the channel function when not previously evident. These observations indicate that over-expression of channels allows for the visualization of changes in channel gating resulting in measurable cytosolic Ca²⁺ signals. This may result from increased clustering of IP₃R channels on the ER membrane possibly allowing for increased amplification of signals through Ca²⁺-induced Ca²⁺ release and interactions with licensing proteins such as KRAP. Among the partially functional channels, different degrees of functionality were evident. Detectable Ca²⁺ release was only evident in a small percentage of hR3 V615M cells at expression levels above or equal to Exo. hR3 and at high [CCh]. Remarkably, when over-expressed, the T1424M hR3 mutation exhibited increased function when compared to WT hIP₃R₃ at similar expression levels such that at lower concentrations of agonist, larger evoked responses were observed. The efficacy of Ca²⁺ release through this mutant was however somewhat reduced because the magnitude of Ca²⁺ release achieved at high [CCh] was diminished. A remaining question is why this mutant uniquely exhibits increased activity compared to WT only when over-expressed? As stated, over-expression might reasonably lead to receptor clusters of increased size with enhanced function. In turn, these channels appear to be inherently poorly functioning, but gating competent channels with a leaky pore and a possibility is that this Ca²⁺ leak sensitizes these channels to low levels of IP₃, ultimately resulting in augmented Ca²⁺ signals at low [CCh]. The observation that significant Ca²⁺ puff activity was observed in cells expressing this variant even without an elevation in IP₃ is consistent with this idea.

Structural changes underlying alterations in channel function of IP₃R1 and IP₃R2 have been proposed for mutations in the SD, LBD, and channel pore. However, the mechanisms underlying the changes in channel function in the regulatory and coupling domain, where two of the neuropathy-associated IP₃R₃ mutations are located, are harder to identify. As the human V615M and T1424M variants are all properly localized and would not be predicted to interfere with ligand binding or directly alter the channel pore structure, the functional effects of these mutations are likely mediated by the disruption of channel modulation by regulatory input or by channel gating mechanisms. Notably, in all mutations the amino acid substitution results in substituition to either a methionine residue or cysteine residue, both containing sulfur moieties. Methionine residues have a unique combination of size, flexibility, and chemistry to act as an effective latch for sulfur-aromatic interactions. Sulfur-aromatic interactions occur in the majority of native protein structures, yet little is known about their roles in ion channels and how this substitution in variants might alter function. Of note, both methionine and cysteine residues are subject to modification by reactive oxygen species (ROS) which have been shown to alter IP₃R activity. Future work may involve investigating whether these mutant residues are modified by ROS.

In contrast to the uncertain mechanisms underlying changes in channel function in the neuropathy-associated IP₃R₃ mutations in the regulatory and coupling domain, the R2524C mutation in IP₃R3 at the junction of the 6th TM and the LNK domain likely directly alters the channel pore, similar to the G2498S mutation in IP₃R2. Based on recently published structures of the IP₃R1 and IP₃R3 pores, the positively charged Arg2524 is proposed to interact with Asp2518 of the neighboring subunit. As a result, mutation of the positively charged Arg2524 to an uncharged Cys would likely alter the structure of the channel pore by hindering its interaction with Asp2518. This may result in a re-arrangement of Arg2524 and Asp2518 that in the absence of ligand binding weakens the hydrophobic interactions that hold the TM region together and thus results in a non-functional, open IP₃R₃ channel, depletion of the ER, and activation of SOCE culminating in an increased basal [Ca²⁺].

Given that the location of individual mutations is widely spread through different domains of the protein, together with the dissimilar effects on IP₃-gated activity, it is not clear whether a common mechanism is
responsible for the leaky channel phenotypes and decreased activity observed. It seems likely that the structural disruption imparted by the amino acid substitution alters both long-distance IP3-induced conformational changes from the LBD to the channel domain to different degrees, but also results in structural changes to the channel domain which dilate the pore without IP3 binding. In the case of V615M and T1424M, in contrast to R2425C, it appears that the pore can be further expanded by the ligation of the LBD. Further high-resolution structural studies will be necessary to define how each mutation results in the altered channel structure.

Since all the mutations are present on a single ITPR3 allele, a question is whether the disease phenotype results from the variants acting in a dominant negative fashion when incorporated into IP3R heterotramers. Our previous study investigating mutants associated with ataxia in IP3R1 or anhidrosis in IP3R2 suggests that this is dependent on the region of the IP3R harboring the mutant, but nevertheless is not necessarily intuitively predictable. For example, incorporation of a mutation in critical residues important for IP3 binding within the LBD in a single monomer results in an inactive tetramer, consistent with the requirement for all LBDs to be occupied with IP3 before channel opening. Since IP3R subtypes readily form heterotetrameric assemblies, this dominant negative scenario likely leads to disabling a large proportion of the complement of IP3R expressed in a particular cell. Clearly, the extent of the disruption of activity by mutations in other regions such as the channel domain depends on how radical the changes in properties of the substituted amino acid are and how inter-subunit interactions are disrupted. For example, somewhat unexpectedly, while a mutation in the selectivity filter of the pore-forming region of the IP3R2 resulted in a dead channel when expressed as a homotetramer, IP3R2 retained significant activity when only two of the monomers harbored the variant. This concatenated receptor approach is powerful because the subunit components of the tetramer can be precisely defined as the cell machinery faithfully only assembles tetrameric IP3R structures without re-arrangement of the linked subunits or forming higher order structures that occurs in other systems. We envision a similar concatenated IP3R3 approach, with defined numbers of mutant monomers within the tetramer will be required to define whether these variants act as dominant-negative subunits to disable function.

Disruption of signaling in Purkinje cells of the cerebellum, which abundantly and almost exclusively express IP3R1 is central to understanding the disturbances in gait characteristic of SCA, pontocerebellar ataxia, and Gillespie Syndrome-associated mutations on IP3R1. We have identified here two distinct molecular effects of neuropathy-associated mutations on IP3R3 function, however, the impacted cell type, which underpins the pathological phenotype remains to be established. The pathophysiology of peripheral neuropathies is generally associated with abnormalities in genes that regulate myelin assembly and axonal transport resulting in demyelination and axonopathy. Mutations resulting in the dysregulation of myelin assembly, cytoskeletal structure, endosomal sorting and cell signaling, proteasome, and protein aggregation, and the mitochondria may result in CMT. In addition to roles in several of the processes that may result in CMT, IP3Rs have also been identified in Schwann cells, with the dominant isoform IP3R3 expressed in distinct regions from the other isoforms in dense patches in the perinodal region in close proximity to connexin 32. It is therefore possible that the unique location or predominant expression of IP3R3 in Schwann cells underlies the etiology of disease. The presence of IP3R3 in this region and its role in the other processes that result in CMT, suggest that further work looking at the downstream effects of these IP3R3 mutations on mitochondrial function, cell structure, propagation of Ca2+ signaling in Schwann cells may be necessary to identify the specific pathophysiological mechanism.

Limitations of this study
The activity of IP3R3 variants in this study was monitored by assessing changes in [Ca2+]i which is an indirect, global readout of the cellular Ca2+ signaling machinery. Further experiments with more direct measurements of IP3R activity, for example using “on nucleus” patch clamp will be necessary to define the biophysical mechanism whereby these disease-associated mutants alter function. Similarly, electrophysiological measurements of SOCE in cells expressing these mutants will define how efficiently the mutants activate Ca2+ influx.

STAR★METHODS
Detailed methods are provided in the online version of this paper and include the following:
- KEY RESOURCES TABLE
RESOURCE AVAILABILITY

Lead contact
Materials availability
Data and code availability

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture

METHOD

Generation of IP3R mutants
Generation of stable HEK cell lines
Cell lysis and SDS-PAGE analyses
Immunocytochemistry and confocal microscopy
Single-cell Ca2+ imaging
Population-based Ca2+ imaging
Detection of Ca2+ puffs using TIRF microscopy

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of SDS-PAGE
Analysis of single-cell Ca2+ imaging
Analysis of population-based Ca2+ imaging
Analysis of Ca2+ puffs using TIRF microscopy

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105523.

ACKNOWLEDGMENTS

The Authors wish to thank members of the Yule lab for helpful comments throughout the course of these studies. The authors wish to thank Dr. Ian Parker (UC Irvine) and Dr. Jeffrey Lock (UC Irvine) for assistance and advice with FLIKA. We thank Dr. Irina Serysheva and Dr. Mariah Baker (University of Texas, Health Science Center at Houston) for helpful insights into the structural consequences of these missense mutations. Funding: This work was supported by the National Institutes of Health Grant NIH/DE0014756 to DIY and F31 NIH/DE030670 to AMW. Research G.B was supported by Foundation Flanders (G094522N) the Research Council of the KU Leuven (AKUL/19/34). GB and DIY are part of the FWO Scientific Research Network CaSign (W0.019.17N; W001422N).

AUTHOR CONTRIBUTIONS

LET, VA, JN, AMW, TRK, JWC, and SM were involved in conducting the investigation. LET and VA were involved in the analysis and visualization of the data. LET and DIY were involved in the conceptualization of the research and writing the original draft. GB, SHB, and AL were involved in project administration providing resources and editing the final draft. DIY and GB acquired the funding for the study.

DECLARATION OF INTERESTS

The authors of this work have no competing interests to disclose.

Received: August 29, 2022
Revised: October 10, 2022
Accepted: November 4, 2022
Published: December 22, 2022

REFERENCES

1. Kerkhofs, M., Seitaj, B., Ivanova, H., Monaco, G., Bultynck, G., and Parys, J.B. (2018). Pathophysiologica consequences of isoform-specific IP3 receptor mutations. Biochim. Biophys. Acta. Mol. Cell Res. 1865, 1707–1717. https://doi.org/10.1016/j. bbamcr.2018.06.004.

2. Terry, L.E., Alzayady, K.J., Furati, E., and Yule, D.I. (2018). Inositol 1, 4, 5-trisphosphate receptor mutations associated with human disease. Messenger 6, 29–44.

3. StreB, H., Irvine, R.F., Berridge, M.J., and Schulz, I. (1983). Release of Ca2+ from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1, 4, 5-trisphosphate. Nature 306, 67–69. https://doi.org/10.1038/306607a0.

4. StreB, H., Bayerdorff, E., Haase, W., Irvine, R.F., and Schulz, I. (1984). Effect of inositol-1, 4, 5-trisphosphate on isolated subcellular fractions of rat pancreas. J. Membr. Biol. 81, 241–253. https://doi.org/10.1007/ b01968717.

5. Berridge, M.J. (1993). Inositol trisphosphate and calcium signalling. Nature 361, 315–325. https://doi.org/10.1038/361315a0.
50. Ohba, C., Osaka, H., Iai, M., Yamashita, S., Suzuki, Y., Aida, N., Shimozawa, N., Takamura, A., Doi, H., Tomita-Katsumoto, A., et al. (2013). Diagnostic utility of whole exome sequencing in patients showing cerebellar and/or vermis atrophy in childhood. Neurogenetics 14, 225–232. https://doi.org/10.1007/s10048-013-0375-8.

51. Fogel, B.L., Lee, H., Deignan, J.L., Strom, S.P., Kantarcı, S., Wang, X., Quintero-Rivera, F., Vila, E., Grody, W.W., Perlman, S., et al. (2014). Exome sequencing in the clinical diagnosis of sporadic or familial cerebellar ataxia. JAMA Neurol. 71, 1237–1246. https://doi.org/10.1001/jamaneurol.2014.1944.

52. Gonzaga-Jauregui, C., Harel, T., Gambin, T., Kousi, M., Griffin, L.B., Francescatto, L., Ozes, B., Karaca, E., Jhangiani, S.N., Baig, S.M., and Dahl, N. (2016). A restricted mutation repertoire of de novo mutations in ITPR1 as a diagnostic tool: a pediatric center’s experience. Front Pediatr. 3, 67. https://doi.org/10.3389/fped.2015.00060.

53. Parolin Schnekenberg, R., Perkins, E.M., Miller, J.W., Davies, W.L., D’Adamo, M.C., Pessia, M., Fares, F., Sima, G., Gillard, E., Hudspith, K., et al. (2015). De novo point mutations in patients diagnosed with ataxic cerebral palsy. Brain 138, 1817–1832. https://doi.org/10.1093/brain/awv117.

54. Sasaki, M., Ohba, C., Iai, M., Hirabayashi, S., Osaka, H., Hirota, T., Saito, H., and Matsumoto, N. (2015). Sporadic infantile-onset spinocerebellar ataxia caused by missense mutations of the insosil 1, 4, 5-trisphosphate receptor type 1 gene. J. Neurol. 262, 1278–1284. https://doi.org/10.1007/s00415-015-7705-8.

55. Valencia, C., Husami, A., Holle, J., Johnson, J.A., Qian, Y., Mathur, A., Wei, C., Ameur, A., and Jhangiani, S.N., and Slominsky, P.A. (2016). ITPR1 gene altered in the opisthotonos mouse. J. Neurol. 263, 1705–1712. https://doi.org/10.1007/s00415-016-8307-0.

56. Gerber, S., Alzyaydy, K.J., Burglen, L., Brémont-Gignac, D., Marchesi, V., Roche, O., Rio, M., Funalt, B., Calmon, R., Durr, A., et al. (2016). Recessive and dominant de novo ITPR1 mutations cause Gillespie syndrome. Am. J. Med. Genet. 98, 981–992. https://doi.org/10.1002/ajmg.a.37962.

57. McIntagart, M., Williamson, K.A., Rainer, K.K., Wheeler, A., Seawright, A., De Baere, E., Verdin, H., Bengtson, L.T., Quigley, A., Rainer, J., et al. (2016). A restricted repertoire of de novo mutations in ITPR1 cause Gillespie syndrome with evidence for dominant-negative effect. Am. J. Hum. Genet. 98, 981–992. https://doi.org/10.1016/j.ajhg.2016.03.018.

58. Prasad, A., Rabionet, R., Espinet, B., Zapata, L., Puiggrós, I., Meler, C., Cuix, A., Carria, C., Trujillo, Y., Ossowski, S., García-Muret, M.P., et al. (2016). Identification of gene mutations and fusion genes in patients with seizures syndrome. J. Invest. Dermatol. 136, 1490–1499. https://doi.org/10.1016/j.jid.2016.03.024.

59. Shadrina, M.I., Shulskaya, M.V., Klyushnikov, S.A., Nikopensius, T., Neis, M., Kivistik, P.A., Komar, A.A., Limborska, S.A., Ilarionshin, S.N., and Slominsky, P.A. (2016). ITPR1 gene p.Val1535Met mutation in Russian family with mild spinocerebellar ataxia. Cerebellum Ataxias 7, 71. https://doi.org/10.1186/s10048-016-0040-8.

60. Barresi, S., Niceta, M., Alfieri, P., Brankovic, V., Piccioni, G., Bruselles, A., Barone, M.R., Cusmai, R., Tartaglia, M., Bertini, E., and Zanni, G. (2017). Mutations in the IRBIT domain of ITPR1 are a frequent cause of autosomal dominant congenital ataxia. Clin. Genet. 91, 86–91. https://doi.org/10.1111/cge.12783.

61. Casey, J.P., Hirouchi, T., Hisatsune, C., Lynch, B., Murphy, R., Dunne, A.M., Miyamoto, A., Ennis, S., van der Spek, N., O’Hic, B., et al. (2017). A novel gain-of-function mutation in the ITPR1 suppressor domain causes spinocerebellar ataxia with altered Ca2+ signal patterns. J. Neurol. 264, 1444–1453. https://doi.org/10.1007/s00415-017-8545-5.

62. Das, J., Lilleker, J., Shereef, H., and Ealing, J. (2017). Missense mutation in the ITPR1 gene presenting with ataxic cerebral palsy: description of an affected family and literature review. Neurol. Neurochir. Pol. 51, 497–500. https://doi.org/10.1016/j.pjnn.2017.06.012.

63. Denti, M.L., Barresi, S., Randelma, M., Bellacchio, E., Alfieri, P., Bruselles, A., Pantaleoni, D., Danieli, A., Jarossi, G., Cappa, M., et al. (2017). Identification of novel and hotspot mutations in the channel domain of ITPR1 in two patients with Gillespie syndrome. Gene 628, 141–145. https://doi.org/10.1016/j.gene.2017.07.017.

64. Hsiao, C.T., Liu, Y.T., Liao, Y.C., Hsu, T.Y., Lee, Y.C., and Soong, B.W. (2017). Mutational analysis of ITPR1 in a Taiwanese cohort with cerebellar ataxias. PLoS One 12, e0187503. https://doi.org/10.1371/journal.pone.0187503.

65. Klar, J., Ali, Z., Farooq, M., Khan, K., Wikström, J., Iqbal, M., Zulficar, S., Faryal, S., Baig, S.M., and Dahl, N. (2017). A missense variant in ITPR1 provides evidence for autosomal recessive SCA29 with asymptomatic spinocerebellar hypoplasia in carriers. Eur. J. Hum. Genet. 25, 848–853. https://doi.org/10.1038/ejhg.2017.54.

66. van Dijk, T., Barth, P., Remenan, L., Appelhof, B., Baas, F., and Pol-The, B.T. (2017). A de novo missense mutation in the insosil 1, 4, 5-trisphosphate receptor type 1 gene causing severe pontine and cerebellar hypoplasia: expanding the phenotype of ITPR1-related spinocerebellar ataxia. Am. J. Med. Genet. 173, 207–212. https://doi.org/10.1002/ajmg.a.37962.

67. Zambonin, J.L., Bellomo, A., Ben-Pazi, H., Everman, D.B., Frazer, L.M., Geraghty, M.T., Harper, A.D., Jones, J.R., Kamien, B., Karmohan, K., et al. (2017). Spinocerebellar ataxia type 29 due to mutations in ITPR1: a
case series and review of this emerging congenital ataxia. Orphanet J. Rare Dis. 12, 121. https://doi.org/10.1186/s13023-017-0672-7.

68. Carvalho, D.R., Medeiros, J.E.G., Ribeiro, D.S.M., Martins, B.J.A.F., and Sobanska, A., Pilch, J., Antczak-Marach, D., Zaremska, A., Zbica, D., Antczak-Marach, D., Zaremba, J., and Sulek, A. (2018). De novo ITPR1 variants are a recurrent cause of early-onset ataxia, acting via loss of channel function. Eur. J. Hum. Genet. 26, 1623–1634. https://doi.org/10.1038/s41431-018-0206-3.

71. Wang, L., Hao, Y., Pu, C., Zhao, J., Zhang, X., Chen, Y., Zhang, H., and Gu, W. (2016). Identification of a splicing mutation in ITPR1 via WES in a Chinese early-onset spinocerebellar ataxia family. Cerebellum 17, 294–299. https://doi.org/10.1177/1353999016658674.

72. Gorski, M.M., Lechci, A., Femia, E.A., La Marca, S., Cairo, A., Pappalardo, E., Lotta, L.A., Artoni, A., and Peyvandi, F. (2019). Complications of whole-exome sequencing for causal gene discovery in primary platelet secretion defects. Haematologica 104, 2084–2090. https://doi.org/10.3324/haematol.2018.204990.

74. Stendel, C., Wagner, M., Rudolph, G., and Klopstock, T. (2019). Gillespie’s syndrome with minor cerebellar involvement and no intellectual disability associated with a novel TPR1 mutation: report of a case and literature review. Neuromotology 50, 382–386. https://doi.org/10.1007/s0039-16931530.

75. Cetani, F., Paridi, E., Aretini, P., Saponaro, F., Borsari, S., Mazoni, L., Apicella, M., Civita, P., La Ferla, M., Caligo, M.A., et al. (2020). Whole exome sequencing in familial isolated primary hyperparathyroidism. J. Endocrinol. Invest. 43, 231–245. https://doi.org/10.1007/s0031-019-01107-5.

85. Nagappa, M., Sharma, S., and Taly, A. B. (2022). Charcot-Marie tooth. In StatPearls.

86. Yoshikawa, F., Iwasaki, H., Michikawa, T., Funuchi, T., and Mikoshiba, K. (1999). Cooperative formation of the ligand-binding site of the inositol 1, 4, 5-trisphosphate receptor by two separable domains. J. Biol. Chem. 274, 328–334.

87. Lock, J.T., Alzayady, K.J., Yule, D.I., and Parker, I. (2018). All three IP3 receptor isoforms generate Ca(2+) puffs that display similar characteristics. Sci. Signal. 11, eaau0344. https://doi.org/10.1126/scisignal.aau0344.
J. Biol. Chem. 295, 18160–18178. https://doi.org/10.1074/jbc.RA120.015683.

97. Alzayady, K.J., Wagner, L.E., 2nd, Chandrasekhar, R., Monteagudo, A., Godiska, R., Tall, G.G., Joseph, S.K., and Yule, D.I. (2013). Functional inositol 1, 4, 5-trisphosphate receptors assembled from concatenated homo- and heteromeric subunits. J. Biol. Chem. 288, 29772–29784. https://doi.org/10.1074/jbc.M113.502203.

98. Nicke, A., Rettinger, J., and Schmalzing, G. (2003). Monomeric and dimeric byproducts are the principal functional elements of higher order P2X1 concatamers. Mol. Pharmacol. 63, 243–252. https://doi.org/10.1124/mol.63.1.243.

99. Furuichi, T., Simon-Chazottes, D., Fujino, I., Yamada, N., Hasegawa, M., Miyawaki, A., Yoshikawa, S., Guénet, J.L., and Mikoshiba, K. (1993). Widespread expression of inositol 1, 4, 5-trisphosphate receptor type 1 gene (Insp3r1) in the mouse central nervous system. Recept. Channels 1, 11–24.

100. Hanemann, C.O. (2001). Hereditary demyelinating neuropathies: from gene to disease. Neurogenetics 3, 53–57. https://doi.org/10.1007/s100480010012.

101. Jerath, N.U., and Shy, M.E. (2015). Hereditary motor and sensory neuropathies: understanding molecular pathogenesis could lead to future treatment strategies. Biochem. Biophys. Acta 1852, 667–678. https://doi.org/10.1016/j.bbadis.2014.07.031.

102. Niemann, A., Berger, P., and Suter, U. (2006). Pathomechanisms of mutant proteins in Charcot-Marie-Tooth disease. NeuroMolecular Med. 8, 217–242. https://doi.org/10.1385/nmm:8.1.217.

103. Toews, J.C., Schram, V., Weerth, S.H., Mignery, G.A., and Russell, J.T. (2007). Signaling proteins in the axoglial apparatus of sciatic nerve nodes of Ranvier. Glia 55, 202–213. https://doi.org/10.1002/glia.20448.

104. Martinez-Gómez, A., and Dent, M.A.R. (2007). Expression of IP3 receptor isoforms at the nodes of Ranvier in rat sciatic nerve. Neuroreport 18, 447–450. https://doi.org/10.1097/WNR.0b013e32805866a6.

105. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

106. Ellefsen, K.L., Settle, B., Parker, I., and Smith, I.F. (2014). An algorithm for automated detection, localization and measurement of local calcium signals from camera-based imaging. Cell Calcium 56, 147–156. https://doi.org/10.1016/j.ceca.2014.06.003.

107. Alzayady, K.J., Chandrasekhar, R., and Yule, D.I. (2013). Fragmented inositol 1, 4, 5-trisphosphate receptors retain tetrameric architecture and form functional Ca2+ release channels. J. Biol. Chem. 288, 11122–11134. https://doi.org/10.1074/jbc.M113.453241.

108. Arige, V., Emrich, S.M., Yoast, R.E., Trebak, M., and Yule, D.I. (2021). A protocol for detecting elemental calcium signals (Ca(2+) puffs) in mammalian cells using total internal reflection fluorescence microscopy. STAR Protoc. 2, 100618. https://doi.org/10.1016/j.xpro.2021.100618.

109. Emrich, S.M., Yoast, R.E., Xin, P., Arige, V., Wagner, L.E., Hempel, N., Gill, D.L., Sneyd, J., Yule, D.I., and Trebak, M. (2021). Omnitemporal choreographies of all five STIM/Orai and IP3Rs underlie the complexity of mammalian Ca(2+) signaling. Cell Rep. 34, 108760. https://doi.org/10.1016/j.celrep.2021.108760.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Purified Mouse Anti-IP$_3$R-3 | BD Transduction | Cat. #610313 |
| GAPDH Monoclonal Antibody (6CS) | Invitrogen | Cat. #AM4300 |
| Goat anti-mouse Dylight™ 800CW secondary antibody | Invitrogen | Cat. #SAS-35521 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Alexa Fluor 488 | Invitrogen | Cat. #A28175 |
| Fura-2/AM | Invitrogen | Cat. # F1221 |
| Cyclopiazonic Acid (CPA) | TOCRIS | Cat. # 1235 CAS #18172-33-3 |
| CRAC Channel Inhibitor IV, GSK-7975a | Sigma Aldrich | Cat. # 53453100001 CAS #1253186-56-9 |
| Cal520-AM | AAT Bioquest | Cat. #21130 |
| ci-IP$_3$/PM | TOCRIS | Cat. #6210 CAS #1009832-82-9 |
| EGTA-AM | Invitrogen | Cat. #E1219 |
| Dulbecco's Modified Eagle Medium (DMEM) | Gibco | Cat. #11995073 |
| Fetal Bovine Serum | Gibco | Cat. #16-000-044 |
| Penicillin/Streptomycin | Gibco/Life Technologies | Cat. #15140122 |
| Geneticin Sulfate (G418) | Gibco/Life Technologies | Cat. #11811031 |
| Pfu Ultra II Hotstart 2X Master Mix | Agilent | Cat. #600850 |
| Pierce™ Protease Inhibitor Tablets, EDTA-free | Thermo Scientific | Cat. #A32965 |
| Paraformaldehyde (PFA) | Sigma Aldrich | CAS#30525-89-4 |
| Bovine Serum Albumin (BSA) | Fisher | Cat. #BP9700100 |
| poly-D-lysine | Sigma Aldrich | CAS#25988-63-0 |
| Carbamoylcholine chloride (Carbachol) | Sigma Aldrich | Cat. #C4382 CAS #51-83-2 |
| **Critical commercial assays** | | |
| D$_2$ protein assay kit | Bio-Rad | Cat. #5000112 |

#### Experimental models: Cell lines

| Cell line | Source | Identifier |
|-----------|--------|------------|
| IP$_3$ Null HEK-293 Cell Line (HEK-3KO) | Kerafast | Cat. # EUR030 |
| HEK293 endogenous hIP$_3$R3 | This Paper | N/A |
| HEK-3KO exogenous hIP$_3$R3 | This Paper | N/A |
| HEK-3KO exogenous hIP$_3$R3 V615M | This Paper | N/A |
| HEK-3KO exogenous hIP$_3$R3 T1424M | This Paper | N/A |
| HEK-3KO exogenous hIP$_3$R3 R2524C | This Paper | N/A |

#### Oligonucleotides

| Primer | Source | Identifier |
|--------|--------|------------|
| hiP$_3$R3 T1424M forward primer: CTGCTAC GTAGACATGGAGGTGAGATG | This Paper | N/A |
| hiP$_3$R3 T1424M reverse primer: CTGCTACG TAGACATGGAGGTGAGATG | This Paper | N/A |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David Yule (David.Yule@urmc.rochester.edu).

**Materials availability**
Plasmids (hIP3R3 V615M, hIP3R3 T1424M, and hIP3R3 R2524C) and cell lines (Endo. hR3, Exo. hR3, and IP3R3 mutants) generated in this study are available upon request.

**Data and code availability**
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture
All cell lines used were derived from female, human embryonic kidney epithelial cells (HEK293) originally obtained from ATCC and their identity was authenticated by genomic sequencing prior to gene editing. HEK-3KO cells, HEK293 cells engineered through CRISPR/Cas9 gene editing for the deletion of the three endogenous IP₃R isoforms⁸⁰,¹⁰⁷ and HEK293 cells modified by CRISPR/Cas9 to only express endogenous hIP₃R3 (Endo. hR3). Gene editing was confirmed by genomic sequencing.⁸⁴ The cells were grown at 37°C with 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco/Life Technologies). HEK-3KO cells stably expressing mutant IP₃R3 (hR3 V615M, hR3 T1424M, and hR3 R2524C) were grown at 37°C with 5% CO₂ in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1.5–2 mg/mL Geneticin sulfate (G418; Gibco/Life Technologies).

METHOD

Generation of IP₃R mutants
A two-step QuikChange mutagenesis protocol was used to introduce amino acid substitutions into cDNA encoding the human IP₃R3 (hIP₃R3; NM_002224.4) in pDNA3.1. Mutagenesis and all DNA modifications were carried out using PFu Ultra II Hotstart 2X Master Mix (Agilent). Mutagenesis primers for hIP₃R3 T1424M forward (CTGCTACGTAGACATGGAGGTGGATG), hIP₃R3 T1424M reverse (CATCTCCACCTCAGTCTACGTAGCAG) were synthesized by Integrated DNA Technologies (IDT). In addition to encoding the specified mutations, primers also silently introduced a restriction site for verification purposes. The coding regions for all constructs were confirmed by sequencing. hIP₃R3 V615M and hIP₃R3 R2524C mutant cDNAs were obtained from Julika Neumann (KU Leuven).

Generation of stable HEK cell lines
Transfection of HEK-3KO cells to exogenously express desired hIP₃R3 WT or mutant constructs stably was performed as previously described.⁸⁰,¹⁰⁷ In brief, 5 million cells were pelleted, washed once with Phosphate Buffered Saline (PBS), and resuspended in a transfection reagent (362.88 mM ATP-disodium salt, 590.26 mM MgCl₂ 6.H₂O, 146.97 mM KH₂PO₄, 23.81mM NaHCO₃, and 3.7 mM glucose at pH 7.4). 4-6 µg of DNA was mixed with the resuspended cells and electroporated using the Amaxa cell nucleofector (Lonza Laboratories) program Q-001. Cells were allowed to recover for 48 h before passage into new 10 cm plates containing DMEM media supplemented with 1.5–2 mg/mL G418. Following 7 days of selection, cell colonies were picked and transferred to 24-well plates containing DMEM media supplemented with 1.5–2 mg/mL G418. Wells that exhibited growth were expanded and those expressing the desired constructs were confirmed by western blotting.

Cell lysis and SDS-PAGE analyses
HEK-3KO cells, Endo. hR1 cells, and HEK-3KO cells stably expressing IP₃R constructs were harvested by centrifugation (200 x g for 5 min), washed once with PBS, and solubilized in membrane-bound extraction lysis buffer containing 10 mM Tris-HCl, 10 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM NaF, 20 mM Na₂P₂O₇, 2 mM Na₃VO₄, 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), and 10% glycerol supplemented with a cocktail of protease inhibitors (Pierce). Lysates were incubated for a minimum of 30 min on ice and cleared by centrifugation (16,000 x g for 10 min) at 4°C. Protein concentrations in cleared lysates were determined using DC protein assay kit (Bio-Rad) and 4x SDS gel loading buffer was subsequently added to 5 µg of lysate. Proteins were resolved using 8% SDS-PAGE and transferred to a nitrocellulose membrane (Pall Corporation). Membranes were probed with a mouse monoclonal antibody against IP₃R3 (R3; #610313, BD Transduction) at a 1:1000 dilution and GAPDH (AM4300, Invitrogen) at a 1:75,000 dilution, followed by the goat anti-mouse DyLight™ 800CW secondary antibodies at a 1:10,000 dilution (SA535521; Invitrogen). Membranes were imaged with an Odyssey infrared imaging system (LICOR Biosciences).

Immunocytochemistry and confocal microscopy
HEK-3KO cells stably expressing WT and mutant IP₃R3 constructs were plated on poly-d-lysine coated coverslips. At roughly 50% confluent, cells were fixed using 4% PFA at room temperature for 10 min. Subsequently, coverslips were washed with PBS, and cells were blocked in 10% Bovine Serum Albumin (BSA) for 1 h. Following blocking, cells were incubated in primary antibody against IP₃R3 (BD Transduction) overnight at 4°C. The following day, the primary antibody was removed, and coverslips were washed 3 times with PBS
for 10 min with gentle rocking. Subsequently, the anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Invitrogen) was incubated for 1 h at room temperature with gentle rocking. After incubation, coverslips were washed with PBS and mounted on slides. After allowing slides to dry, coverslips were sealed onto slides and imaged using confocal microscopy using an Olympus Fluoview 1000 microscope.

**Single-cell Ca²⁺ imaging**

Single-cell Ca²⁺ imaging was performed in intact cells as described previously. Glass coverslips were plated with HEK-3KO, Endo.hR3, Exo.hR3, or HEK-3KO cells stably expressing IP₃R mutant constructs at least 18 h prior to imaging experiments. Subsequently, the glass coverslips were mounted onto a Warner chamber and the cells were loaded with 2 μM Fura-2/AM (Invitrogen) in Ca²⁺-containing imaging buffer (Ca²⁺ IB): 10 mM HEPES, 1.26 mM Ca²⁺, 137 mM NaCl, 4.7 mM KCl, 5.5 mM glucose, 1 mM Na₂HPO₄, 0.56 mM MgCl₂, at pH 7.4) at room temperature for 25 min. Following loading, cells were perfused with Ca²⁺ IB which provided a basal 340/380 ratio of the [Ca²⁺]. To obtain measurements of agonist-induced Ca²⁺ release into the cytoplasm cells were stimulated with 0.3–100 μM CCh. To interrogate the leakiness of the expressed IP₃R, perfusion was switched from Ca²⁺ IB and Ca²⁺-free imaging buffer (Ca²⁺-free IB; 10 mM HEPES, 137 mM NaCl, 4.7 mM KCl, 5.5 mM glucose, 1 mM Na₂HPO₄, 0.56 mM MgCl₂, 1 mM EGTA at pH 7.4) and in the absence of extracellular Ca²⁺, cells were treated with 30 μM CPA (TOCRIS) to measure ER Ca²⁺ content. Finally, 10 μM GSK-7975a (Sigma Aldrich) was used to probe the contribution of Orai to cytosolic [Ca²⁺].

Ca²⁺ imaging was performed using an inverted epifluorescence Nikon microscope with a 40x oil immersion objective. Cells were alternately excited at 340 and 380 nm, and emission was monitored at 505 nm. Images were captured every second with an exposure of 15 ms and 4x4 binning using a digital camera driven by TILL Photonics software. Image acquisition was performed using TILLvisION software and data was exported to Microsoft Excel where means were calculated.

**Population-based Ca²⁺ imaging**

Population-based fluorescence imaging was carried out using a FlexStation3 (Molecular Devices), a high-throughput benchtop optical system utilizing micro-fluidics. HEK-3KO cells or HEK-3KO cells stably expressing IP₃R constructs were loaded with 4 μM Fura-2/AM in complete DMEM media. After 1 h, cells were harvested and subsequently washed, resuspended in Ca²⁺ IB, counted, and dispensed into a black-walled flat-bottom 96-well plate (~300,000 cells/well). The plate was centrifuged (200 x g for 2 min) and placed at 37°C for 30 min prior to commencing the assay. Ca²⁺ IB and varying concentrations of CCh (0.1–100 μM CCh) were added to induce Ca²⁺ release through the IP₃R. Excitation of cells loaded with Fura-2/AM alternated between 340 and 380 nm and emission 510 nm. The total experiment time was set for 200 s, with readings taken every 4 s (~51 readings/run). Data from SoftMax® Pro Microplate Data Acquisition and Analysis software was exported to Microsoft Excel where appropriate ratios, normalizations, and means were calculated.

**Detection of Ca²⁺ puffs using TIRF microscopy**

HEK-3KO cells exogenously expressing WT or mutant hIP₃R3 were cultured on 15-mm glass coverslips coated with poly-D-lysine (100 μg/mL) in a 35-mm dish for 36 h. Prior to imaging, the cells were washed three times with imaging buffer. The cells were subsequently incubated with Cal520-AM (5 μM; AAT Bioquest #21130) and ci-IP₃/PM (0.1 or 1 μM, Tocris #6210) in imaging buffer with 0.01% BSA in dark at room temperature. After 1-h incubation, the cells were washed three times with imaging buffer and incubated in imaging buffer containing EGTA-AM (5 μM, Invitrogen #E1219). After 45 min incubation, the media was replaced with fresh imaging buffer and incubated for additional 30 min at room temperature to allow for de-esterification of loaded reagents. Following loading, the coverslip was mounted in a chamber and imaged using an Olympus IX81 inverted Total Internal Reflection Fluorescence Microscope (TIRFM) equipped with oil-immersion PLAPO TIRFM 60x objective lens/1.45 numerical aperture. Olympus CellSens Dimensions 2.3 (Build 189,987) software was used for imaging. The cells were illuminated using a 488 nm laser to excite Cal-520 and the emitted fluorescence was collected through a band-pass filter by a Hamamatsu ORCA-Fusion CMOS camera. The angle of the excitation beam was adjusted to achieve TIRF with a penetration depth of ~140 nm. Images were captured from equal areas using a 4 X 4 or 2 X 2 pixel binning (433.333 or 216 mm/pixel) at a rate of ~166 or ~50 frames/second by directly streaming into RAM. To photo-release IP₃, UV light from a laser was introduced to uniformly illuminate the field of view. Both the intensity
of the UV flash and the duration (1 s) for uncaging IP3 were optimized to prevent spontaneous puffs in the absence of loaded ci-IP3. Images were exported as vsi files.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Quantification of SDS-PAGE**
Membranes were quantified using Image Studio Lite (LICOR Biosciences), while mean of at least 3 experiments (n = 3) and SEM were calculated using GraphPad Prism (GraphPad).

**Analysis of single-cell Ca\(^{2+}\) imaging**
In Microsoft Excel mean basal [Ca\(^{2+}\)], change in 340/380 amplitude, and percentage of responding cells for each experiment (multiple cells) was calculated. Area under the curve (AUC) was calculated using GraphPad Prism as increases above baseline that are greater than 10% of the distance from the minimum to the maximum Y using the 20 data points prior to agonist addition as the baseline. The mean and SEM of at least n = 3 experiments for each cell line was calculated in GraphPad Prism and statistical analysis was performed using one-way ANOVA with Tukey’s test.

**Analysis of population-based Ca\(^{2+}\) imaging**
In Microsoft Excel, peak amplitude of CCh-induced Ca\(^{2+}\) release was calculated by normalizing the 340/380 ratio to the average of the first 5 340/380 ratio data points. Area under the curve (AUC) was calculated using GraphPad Prism as increases above baseline that are greater than 10% of the distance from the minimum to the maximum Y using the five data points prior to agonist addition as the baseline. Means and SEM from at least 3 individual plates and nonlinear curve fits were calculated in GraphPad Prism. Statistical analysis was performed in GraphPad Prism using one-way ANOVA with Tukey’s test.

**Analysis of Ca\(^{2+}\) puffs using TIRF microscopy**
Images, 5 s prior and 30–60 s after flash photolysis of ci-IP3, were captured, as described previously.\(^{108,109}\) The vsi files were converted to tiff files using Fiji\(^{105}\) and further processed using FLIKA, a Python programming-based tool for image processing.\(^{106}\) From each recording, ~100 frames (~2 s) before photolysis of ci-IP3 were averaged to obtain a ratio image stack (F/F\(_0\)) and standard deviation for each pixel for recording up to 30 s following photolysis. The image stack was Gaussian-filtered, and pixels that exceeded a critical value (1.0 for our analysis) were located. The ‘Detect-puffs’ plug-in was utilized to detect the number of clusters (puff sites), number of events (number of puffs), amplitudes and durations of localized Ca\(^{2+}\) signals from individual cells. All the puffs identified automatically by the algorithm were manually confirmed before analysis. The results from FLIKA were saved to Microsoft Excel and graphs were plotted using GraphPad Prism 8 where the mean and SEM were calculated for at least 3 individual experiments. Statistical analysis was performed in GraphPad Prism using a two tailed t-test.