Phasor FLIM-FRET Analysis of Voltage Sensor Conformational Heterogeneity in hERG Potassium Channels

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ABSTRACT

Voltage sensors are crucial for the electromechanical coupling of rapid delayed rectifier hERG channels in cardiac pacemaking. They detect and respond to changes in membrane voltage by traversing across the membrane electric field. Mutations in voltage-sensing arginines of hERG associated with Long QT syndrome induce distinct channel gating behaviors, yet knowledge about voltage sensing in these mutants remains limited. The goal of this research is to elucidate potential intermediate states and conformational heterogeneity of voltage sensors, by leveraging patch-clamp electrophysiology, fluorescence lifetime imaging microscopy (FLIM), and transition metal Förster resonance energy transfer (tmFRET). Facilitated by dual stop-codon mediated noncanonical amino acid incorporation, along with click chemistry for site-specific labeling, we preformed FLIM-tmFRET measurements on the hERG voltage sensor. Analysis of lifetime changes of the FRET donor L-acridonyl-alanine (Acd) using the phasor approach of FLIM revealed multiple FRET states in mutant hERG channels, in contrast to the single high FRET state observed in wild-type hERG channels. The presence of these intermediate FRET states correlates with the specific location of mutations, suggesting distinct voltage-sensor conformations. This study provides novel insights into cardiac channelopathies and the underlying structural mechanisms of voltage sensing in cardiac arrhythmias.

INTRODUCTION

Voltage sensing of ion channels is crucial for regulating cardiac and neuronal excitability[1]. Voltage sensors detect changes in membrane voltage, initiating conformational changes that govern the opening and closing of voltage-gated ion channels (VGICs)[1,2]. This pivotal role allows VGICs to precisely control the flux of ions across cell membranes, thereby orchestrating essential physiological processes such as action potentials in neurons, muscle contraction, cardiac pacemaking, and secretion of neurotransmitters. Notably, dysfunction in ion channels is implicated in various diseases, including the prolonged QT-interval syndrome (long QT syndrome, LQTS), a cardiac disorder characterized by prolonged ventricular repolarization, which can cause life-threatening arrhythmias. Mutations in the human ether-à-go-go-related gene (hERG), which encodes the cardiac potassium channel Kv11.1, represent a prevalent cause of congenital LQTS[3,4]. The hERG channel, belonging to the voltage-gated ion channel (VGIC) superfamily, features a functional voltage sensor, where mutations linked to LQTS have been identified[5].

Voltage sensors of VGICs are characterized by repeats of positively charged arginines within the 4th transmembrane helix (S4 helix), which directly sense transmembrane electric field[2]. The function of the S4 helix relies on significant "up and down" movements[2,6-12]. The precise conformational changes associated with these movements are critical for channel gating in response to electrical fluctuations across the membrane, a process referred to as electromechanical coupling. High-resolution structures capturing the "down" conformation, corresponding to the S4 voltage sensor at resting hyperpolarized voltages, were first observed in an *Arabidopsis* two-pore channel (TPC)[6]. Similar "down" conformations have been identified in other channels such as voltage-gated sodium channels and pacemaker hyperpolarization-activated cyclic nucleotide-gated (HCN) channels[7-10,13]. Moreover, cryo-electron microscopy (cryo-EM) studies have provided atomic-level insights into the voltage sensors of ether-à-go-go (Eag) channels, a close relative of hERG, revealing multiple states including up, down, and intermediate conformations[11]. However, understanding the functional significance of these intermediate states remains a challenge. A more comprehensive understanding of the structural dynamics and potential multi-step movements of the voltage sensor is crucial for elucidating the electromechanical coupling mechanisms governing VGIC function.

While fluorescence imaging stands as a valuable tool for understanding the structural mechanisms of voltage sensors, significant technical hurdles arise in controlling membrane voltages for such imaging experiments. Single-molecule fluorescence techniques hold promise, with their capacity to discern distinct protein conformational states, yet their application in probing the voltage-dependent

structural arrangements of VGICs remains limited. Understanding the structure-function relationship of VGICs often necessitates live-cell imaging, particularly as voltage sensor dynamics depend on membrane lipids in their native environment. Traditional fluorescence intensity-based ensemble imaging, while informative, tends to homogenize signals when assessing numerous molecules within living cells. Conversely, emerging fluorescence lifetime-based imaging methodologies, such as fluorescence lifetime imaging microscopy (FLIM), have great potential to effectively dissect diverse populations of measured molecules exhibiting different conformations[14-17].

In this study, we employed a combination of techniques including nonsense suppression-mediated incorporation of noncanonical amino acids for site-specific fluorescence labeling, FLIM-based transition metal Förster resonance energy transfer (FLIM-tmFRET), and patch-clamp electrophysiology to examine the impact of a LQTS-associated mutation on conformational rearrangements of hERG voltage sensors in transfected cell lines. We designed a protocol to study the conformational dynamics of voltage sensors by analyzing the frequency-domain phasor plot function of FLIM-tmFRET. This methodology has significant potential for broad applications, offering an effective approach to quantitatively assess the movement, states, dynamics, and energetics of voltage sensors in ion channels.

RESULTS

Functional impact of S4 helix LQTS mutations on hERG channel gating

The hERG (K_v11.1 or KCNH2) channels expressed in tsA-201 cells exhibit a depolarization-dependent activation profile like other voltage-gated potassium channels. Within the S4 helix of hERG, six positively charged arginines or lysines are present, with the arginines likely serving as the primary voltage-sensing residues (Supplementary Fig. 1a). Notably, mutations of R531 and R534 have been associated with LQTS[5,18].

We generated homology models (SWISS-MODEL) of hERG based on previous findings identifying three distinct conformations of rat Eag channel voltage sensors using cryo-EM techniques[11,19]. In the down state model at hyperpolarized voltages (Supplementary Fig. 1b), only the side chain of K525 of hERG is positioned above the "phenylalanine cap" F463 at the hydrophobic constriction site. Upon membrane depolarization, the side chains of K525, R528, and R531 are predicted to relocate above the phenylalanine cap. In comparison, in the model of the hypothetical

intermediate state, the side chains of K525 and R528, but not that of the R531, are positioned above the phenylalanine cap. We postulate that this intermediate state may represent an important point along the trajectory of the voltage sensor movement within hERG channels.

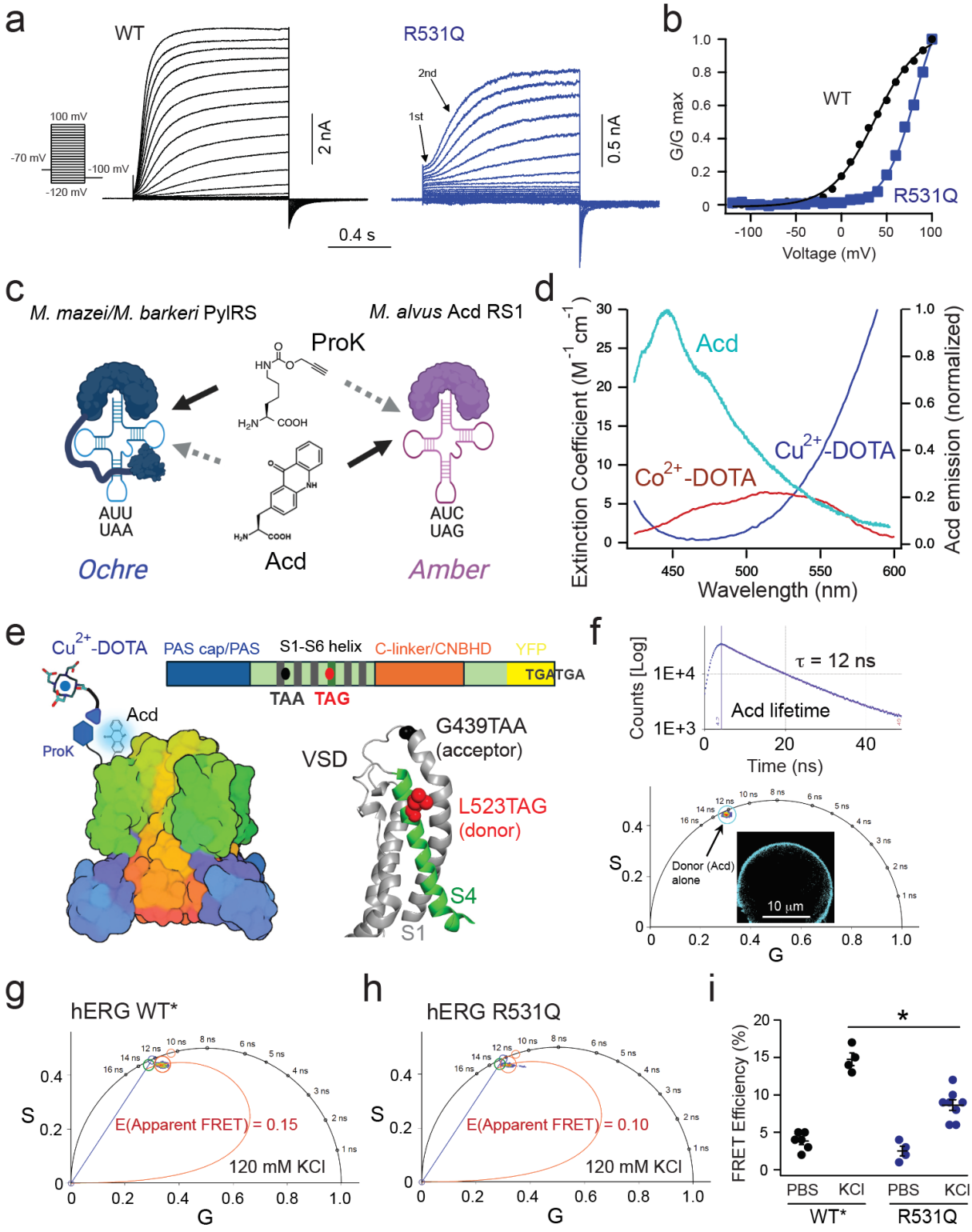

Fig. 1. Effects of the R531Q mutation on hERG gating and the design of phasor FLIM-tmFRET strategies for measuring voltage sensor movement in WT and R531Q hERG channels.

a. Representative current traces for activation of wild-type (WT) hERG1 channels, elicited by steps of depolarizing voltage pulses from -120 mV to +100 mV. Tail currents were recorded at -100 mV. Also shown are representative current traces for activation of hERG1-R531Q channels, produced using the same voltage protocol.

b. Comparison of conductance-voltage (G-V) relationships for activation of hERG WT and hERG-R531Q channels.

c. Diagram illustrating the dual stop-codon (amber and ochre) suppression for incorporating Acd and ProK in a mutually orthogonal manner.

d. Absorption and emission spectra showing the spectral overlap between the Acd emission incorporated to hERG channels (L523 site) and the molar absorption of Co^{2+} or Cu^{2+} coordinated with azido-DOTA.

e. Design of a tmFRET pair with the FRET donor positioning in the L523TAG site of the S4 helix and the FRET acceptor in the C-terminal end of the S1 helix (near the S1-S2 linker).

f. Mono-exponential lifetime decay of Acd when incorporated into the L523 site. The lifetime species of membrane localized Acd can also be displayed in the phasor plot, with $\tau(\varphi)$ = 11.71 ns and $\tau(\varphi)$ = 12.64 ns. The slight deviation of the Acd lifetime species from the universal semicircle is due to background contribution, typically 2% - 5%.

g. FRET trajectory calculation enabled by the phasor plotting for hERG WT* channels (hERG-WT with only the L523TAG and G439TAA mutations), labeled with Cu^{2+} acceptor via CuAAC click chemistry in the presence of 120 mM extracellular KCl.

h. Similar FRET trajectory calculation for hERG-R531Q channels as a comparison.

i. Summary data showing the apparent FRET efficiency of hERG WT* and hERG R531Q channels, either in regular PBS solution or with 120 mM KCl, using the FRET trajectory method as in panels g and h. Data shown are mean ± s.e.m., n = 4 – 8, *p < 0.05.

In the presence of 10 mM tetraethylammonium (TEA) to fully inhibit endogenous potassium currents in tsA cells[20] (Supplementary Fig. 1c) - different from the wild-type (WT) hERG channels[21] - LQTS-associated hERG-R531Q channels demonstrated a noticeable initial phase of approximately 100 ms, preceding the delayed second phase (Fig. 1a). This delay in hERG currents demonstrates the functional impact of the R531Q mutation in LQTS development. Examining the tail current amplitude in relation to applied voltages revealed a substantial rightward shift in the conductance-voltage (G-V) relationship compared to that of wild-type channels (Fig. 1b); the voltage of the half maximal activation for the R531Q mutant channel was +80 ± 3.7 mV (n = 4) compared to +35 ± 4 mV (n = 3) for the WT channel. This approximately 45 mV shift in the G-V and the biphasic channel activation are consistent with prior research and indicative of potential intermediate functional states for the R531Q mutant[18]. Furthermore, this initial delay was shorter in hERG-R528Q channels, followed by a double-exponential main current phase for the activation (Supplementary Fig. 1d); the deactivation was much slower. The R528Q mutation does not change the G-V relationship compared to the WT and has not been linked to LQTS. Additionally, LQTS-linked mutations R534L or R534Q produced hERG channels that lack the multi-phasic activation kinetics, instead displaying noticeable voltage-induced channel inactivation (Supplementary Fig. 1e). Assessing the functional impact of these three charge-neutralization mutations on channel activation, the R531Q produces the most significant effect.

Measuring the movement of voltage sensors in hERG channels using FLIM-tmFRET

To advance our structural investigation of the impact of LQTS mutations on hERG gating, we implemented a dual stop-codon suppression strategy to incorporate noncanonical amino acids for site-specific fluorescence labeling and FRET measurements of voltage-sensor movements[22]. We modified a human hERG1 construct by introducing amber (TAG) and ochre (TAA) stop codons at selected positions within the voltage-sensing domain (VSD) of hERG channels (Fig. 1). The amber stop-codon suppression approach facilitated the incorporation of a fluorescent noncanonical amino acid L-acridonyl-alanine (Acd) into the S4 helix[23,24]. This was achieved using a recently developed Acd tRNA/tRNA synthetase system adapted from the pyrrolysyl-tRNA synthetase of *M. alvus* archaea[24] (Fig. 1c). Importantly, this orthogonal strategy operates independently of the ochre stop-codon suppression system, which is derived from the pyrrolysyl-tRNA synthetase of *M. mazei*/*M. barkeri* archaea[25]. Specifically, we used a tRNA (M15-PylT)/PylRS pair derived from *M. mazei*/*M. barkeri* to introduce N-propargyl-L-lysine (ProK) into another relatively immobile site within the VSD. ProK features an alkyne group capable of undergoing specific chemical reactions with azide-containing compounds via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry. Previously, we have applied a similar approach to perform FLIM-tmFRET experiments for HCN channels[22,25], with the integration of Acd marking a notable enhancement over our prior dual stop-codon suppression methodology. Furthermore, this protein labeling design circumvents the necessity for cysteine-based labeling, mitigating potential concerns of mutating endogenous cysteine residues within hERG channels.

We designed a tmFRET pair comprising Acd as the FRET donor and Cu^{2+} chelated by 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) as the FRET acceptor. The emission spectrum of Acd overlaps with the absorption spectrum of Cu^{2+} or Co^{2+} coordinated by an azido mono-amide-DOTA (Fig. 1d). The weak molar absorption of transition metals results in a shorter Förster distance (R_0 = ~ 10 – 20 Å, depending on the exact design) for tmFRET compared to conventional FRET pairs[16,17,26-28]. This reduced working distance of tmFRET is well-suited for investigating voltage-sensor movement in ion channels[9,10,22,29]. For probing the S4 movement of hERG channels, we positioned the Acd site at the L523 position near the N-terminal side of the S4 helix and the ProK/Cu^{2+}-DOTA site at the G439 position at the C-terminal end of the S1 helix (Fig. 1e). This design is predicated on the premise that the FRET donor and acceptor should be distant enough when the S4 is in the down state, resulting in minimal FRET, and exhibit increased FRET when they approach due to membrane depolarization. Because of the short R_0 of this tmFRET pair, FRET occurs primarily within the same subunit; inter-subunit FRET is negligible. Additionally, we fused a YFP to the C-terminal end of the channel to indicate

the expression of full-length proteins at the cell membrane. Our observations revealed a correlation between YFP fluorescence and Acd fluorescence, confirming the expression of hERG channels (Supplementary Fig. 2).

Phasor FLIM was used to quantify the tmFRET by measuring the lifetime of Acd in tsA cells. One advantage of Acd is its exceptionally long and mono-exponential lifetime (~16 ns free in solution and ~12 ns when incorporated in cells)[16]. Both time-domain and frequency-domain types of FLIM were performed, which yielded similar lifetime values for the membrane-localized fluorescence coming from the Acd incorporated at the L523 position of the S4 helix (Fig. 1f). In the phasor plot of frequency domain FLIM, the lifetime data for each pixel in the image were translated on a Cartesian coordinate, delineated by a universal semicircle (Fig. 1f). Mono-exponential lifetimes precisely aligned with the semicircle, whereas multiexponential lifetimes localize within it. Shorter lifetimes demonstrate a clockwise movement along the semicircle. Considering a small amount of background contribution, the measured Acd lifetime in cells is close to mono-exponential, which is necessary for our subsequent FRET analysis.

We used the FRET trajectory function facilitated by the phasor approach of FLIM to assess the change in tmFRET, comparing tsA cells expressing hERG channels imaged in either regular phosphate-buffered saline (PBS) or a depolarizing solution containing 120 mM KCl (Fig. 2 g-i). This function relies on the concept that the degree of shortened donor lifetime reports FRET efficiency, thereby representing the distance between the FRET donor and acceptor. The FRET trajectory starts from zero FRET efficiency, aligning with the donor-alone reference, and follows a path through the measured phasor species, ending at the theoretical 100% FRET efficiency at the origin (0, 0) coordinate, where most of the background noise is situated (Supplementary Fig. 2b). For hERG-G439TAA/L523TAG-YFP channels (referred to as WT* denoting no additional mutations), FRET efficiency was minimal in PBS as expected, but increased upon exposure to 120 mM KCl. In addition, treatment of 120 mM KCl did not affect the lifetime of the Acd donor alone. Conversely, in comparison to WT*, the presence of R531Q in the hERG construct produced a similarly low FRET efficiency in PBS, but the increase in FRET after the high KCl was attenuated (Fig. 2i). This outcome corresponds to the rightward shift observed in the G-V curve of the hERG-R531Q channels, indicating a compromise in the extent or likelihood of voltage sensor movement.

However, while the FRET trajectory method used in ensemble imaging experiments offers valuable insights, it also comes with limitations. Although it provides a quantified FRET efficiency, it only

represents an averaged distance between donors and acceptors, lacking the capacity to reveal more detailed structural information and the conformational heterogeneity of the S4 helix. This limitation hinders its ability to effectively test hypotheses regarding the presence of intermediate states of hERG channel voltage sensors and evaluating the potential impact of the R531Q mutation on distinct conformational states of the voltage sensor. Moreover, FRET trajectory is often inadequate for fitting the distribution of measured lifetime species in the phasor plot. To better interpret data, we used an alternative approach, leveraging phasor plot analysis of FLIM[14,30].

Probing the conformational heterogeneity of hERG voltage sensors using the phasor approach of FLIM

This alternative analysis leverages the linear unmixing property of the FLIM phasor plot[14,15,30]. We consider a voltage sensor with two states: up and down states. The down-state model of the S4 corresponds to the low FRET state, while the up-state model corresponds to the high FRET state (Fig. 2a). In our tmFRET design, the low FRET state, occurring when the distance between the donor and acceptors exceeds ~25 Å, yields nearly negligible FRET, which essentially makes the donor lifetime nearly unchanged compared to the donor alone situation. Consequently, the measured FRET comprises a mixture of low and high FRET states, and the location of the measured lifetime is primarily determined by the probability of the voltage sensor being in the high FRET state.

To demonstrate linear unmixing in the phasor plot, we used cells expressing the hERG-L523TAG/G439TAA construct, dually labeled with Acd and Cu^{2+}-DOTA (Fig. 2b). Under depolarizing conditions achieved with 120 mM external KCl, the measured FRET can be resolved into two contributors: the donor alone and the position of lifetime if 100% of the voltage sensors are in the high FRET state (Fig. 2b). The linear fitting depends on the pattern of lifetime distribution, resembling a linear trend traversing the major axis of the 'oval' shape formed by lifetime species originating from membrane-localized pixels in the phasor plot (Fig. 2b). This phenomenon likely emerges from the conformational heterogeneity among numerous channel molecules, each adopting various S4 conformational states. In this context, a more pronounced major axis (greater length-to-width ratio) of the oval-shaped lifetime distribution may suggest a greater diversity in the population of FRET donor-acceptor distances. The measured high FRET value of 0.53, corresponding to a converted distance of approximately 13 Å, reflects the FRET efficiency of the high FRET state, and an estimation of the donor-acceptor distance in the up-state model. As expected, membrane depolarization elevated the proportion of the high FRET state; about 35% of voltage sensors transition to the up state, aligning with

the relatively low open probability of wild-type hERG channels at approximately +10 mV, a potential achievable with 120 mM KCl, as previously demonstrated using voltage-sensitive dyes[22] (Fig. 2b).

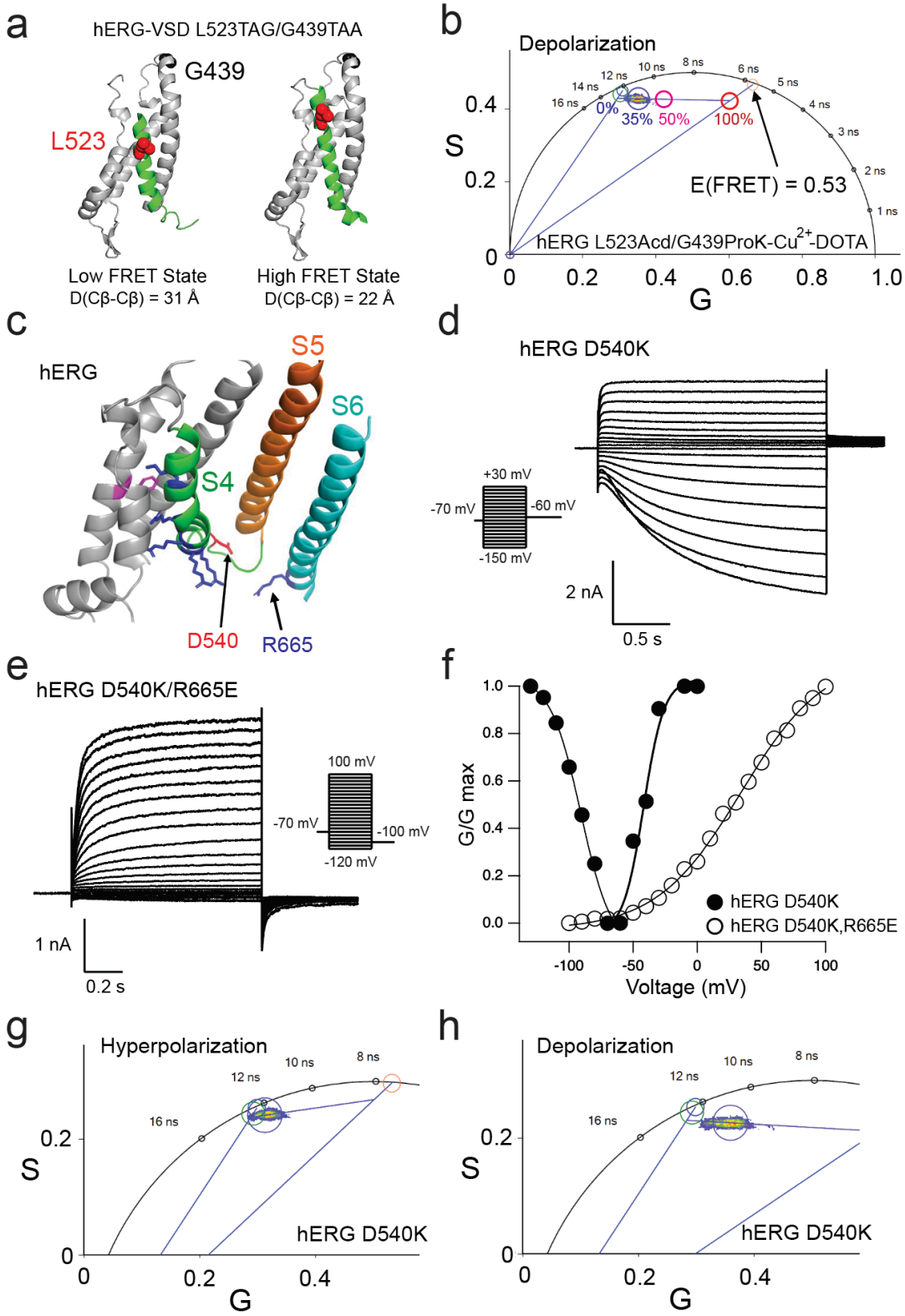

Fig. 2. Applying linear unmixing of the phasor analysis to the FLIM-tmFRET for hERG channels.
a. Structural cartoons illustrating the low FRET state and high FRET state in the context of L523TAG/G439TAA FRET pair, corresponding to the homology models of hERG-VSD in the down and up states. Distances between β carbons, D(Cβ-Cβ), of L523 and G439 are shown as well. The low FRET state yields negligible FRET.
b. Representative linear unmixing approach to estimate the FRET efficiency corresponding to the high FRET state. The fitting is based on the pattern of lifetime distribution on the phasor plot. The red cursor points to a hypothetical scenario that 100% of voltage sensors are in the up position, and thus the high FRET state. With depolarization via 120 mM KCl, the percentage of voltage sensors in the high FRET state is 35%. The background contribution is 4% for the donor alone (0% FRET) lifetime and 6% in the presence of the acceptor Cu^{2+}-DOTA.
c. Structural cartoon (upward view) showing the positions of D540 and R665 within the hERG channel.
d-e. Representative current traces for activation of hERG-D540K and hERG-D540K/R665E charge-swapped channels.
f. Comparison of conductance-voltage (G-V) relationships for activation of hERG-D540K and hERG-D540K/R665E channels.
g-h. Combined fluorescence signals from four cells for linear unmixing fitting of hERG-D540K channels in either hyperpolarizing or depolarizing voltage conditions. In the hyperpolarizing condition achieved with extracellular 107 mM NMDG[31], the same tmFRET strategy was employed for analysis. Different FRET efficiencies exhibited when comparing the results from the hyperpolarizing (panel g, FRET efficiency = 0.39) and depolarizing (panel h, FRET efficiency = 0.56) conditions.

We also tested a tmFRET pair using the hERG L523TAG/A430TAA construct (Fig. 3). In this case, even in the down state of S4, the proximity of the donor and acceptor resulted in noticeable FRET signals. As a result, the Acd lifetime distribution displayed a distinct arc shape, contrasting with the oval-shaped pattern observed in the hERG L523TAG/G439TAA construct. The primary component of the distribution (towards shorter lifetime), after a linear fitting, yielded a notably higher FRET efficiency of approximately 0.65 (a converted distance of 10 Å) compared with that of the L523/G439 pair, indicative of a closer donor-acceptor proximity in the L523/A430 pair (Fig. 3). In addition, the minor component of the lifetime distribution exhibited a lower FRET efficiency of 0.37 (as indicated by dashed line fitting in Fig. 3 b-d, corresponding to a converted distance of 16 Å), presumably corresponding to the FRET in the down state of S4. Upon membrane depolarization, there was a gradual increase in the probability of high FRET, suggesting the upward movement of S4. The existence of this minor component posed challenges for accurately fitting the lifetime distribution linearly, particularly if in the presence of additional intermediate FRET efficiencies. Nevertheless, these results emphasize the ability of our FLIM-tmFRET strategy to reliably report S4 movement, with FLIM-tmFRET measurements showing dependence on both donor/acceptor proximity and voltage changes. Since linear unmixing analysis is more effective for a two-state model, particularly when one state shows negligible FRET as seen in the L523/G439 FRET pair, we decided to use the hERG L523/G439 pair for subsequent studies.

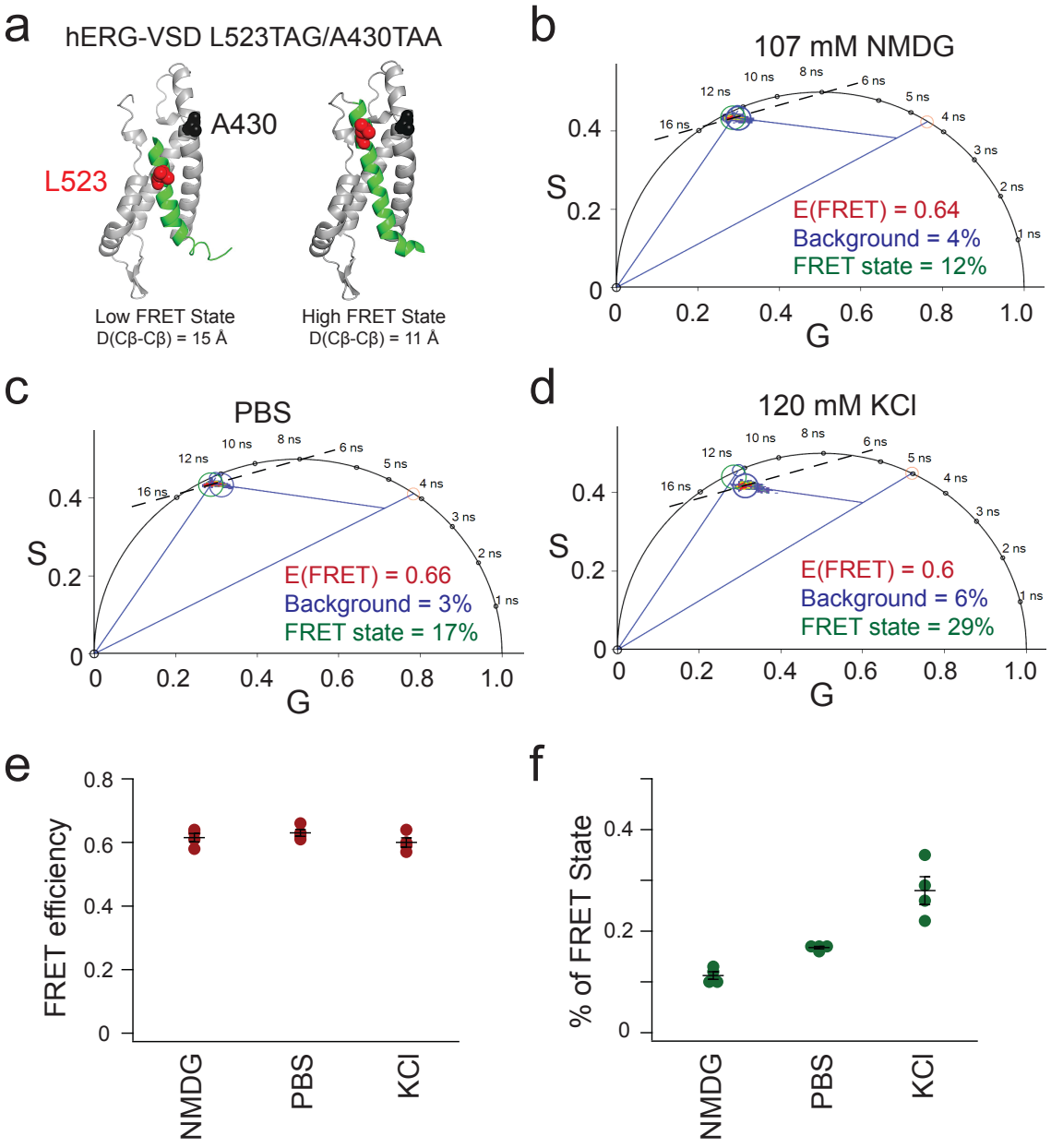

Fig. 3. FLIM-tmFRET on the hERG-L523TAG/A430TAA construct produced arc-shaped lifetime distribution in the phasor plot.

a. Structural cartoons illustrating the low FRET state and high FRET state in the context of L523/A430 FRET pair, corresponding to the homology models of hERG-VSD in the down and up states. Different from the L523/G439 pair, the low FRET state corresponding to the S4 down conformation yields noticeable FRET.

b-d. Representative analysis of individual cells under various conditions indicated. The lifetime distribution has an arc shape. The linear fitting shown is through the shorter-lifetime (major) component, generating a high FRET efficiency around 0.65. The dashed line goes through the longer-lifetime (minor) component, generating a fixed low FRET efficiency around 0.37.

e and f. Summary data of estimated FRET efficiencies and percentage of the high FRET state using linear unmixing of the phasor approach. Data shown are presented as mean ± s.e.m., n = 4.

We initially conducted proof-of-principle experiments employing phasor analysis to validate the efficacy of this approach. We focused on a single mutation, D540K, which induces a "U-shaped" G-V relationship[32]. Notably, hERG-D540K channels exhibit pronounced hyperpolarization-activated currents reminiscent of pacemaker HCN channels, alongside depolarization-activated currents (Fig. 2 c-f). This unique G-V profile suggests the presence of multiple conformations of the S4 voltage sensor. In our structural model, the D540K mutation disrupts a critical salt bridge between D540 at the C-terminal end of the S4 and R665 in the C-terminal end of the S6 helix (Fig. 2c). This disruption leads to the decoupling of the S4 and S6, resembling the structural arrangement observed in HCN channels, where the S4 presumably relies on coupling with the S5 helix rather than with the S6 helix for hyperpolarization-dependent activation[9,10,33]. To restore the wild-type behavior of the channel, we engineered a charge-swapping mutant, hERG-D540K/R665E (Fig. 2 e-f), to recreate the salt bridge and thereby reestablish the S4-S6 coupling[32]. This intervention successfully rescued the native function of hERG channels.

Introducing the D540K mutation to engineered hERG channels with the L523/G439 tmFRET pair revealed distinct FRET efficiencies under hyperpolarized versus depolarized membrane conditions (Fig. 2 g-h and Fig 4). In the absence of D540K (hERG-WT*) or with the combined D540K/R665E mutations, only a single high-FRET state was detected, regardless of voltage (Fig. 4 a and c). Increasing the membrane voltage led to a higher proportion of the high-FRET state, with little impact on the amplitude of the FRET efficiency (Fig. 4 a, c, f). These results suggest that for hERG WT* channels, there exist only two major S4 conformations, lacking intermediate ones. D540K mutation introduces additional intermediate S4 conformations, likely contributing to the U-shaped gating behavior. D540K/R665E restores the ‘all or none’ S4 movement in WT* channels, further supporting our interpretation of a direct impact on S4 movement by D540K. This comparative analysis highlights the capability of FLIM phasor analysis in detecting potential intermediate conformations of the voltage sensor.

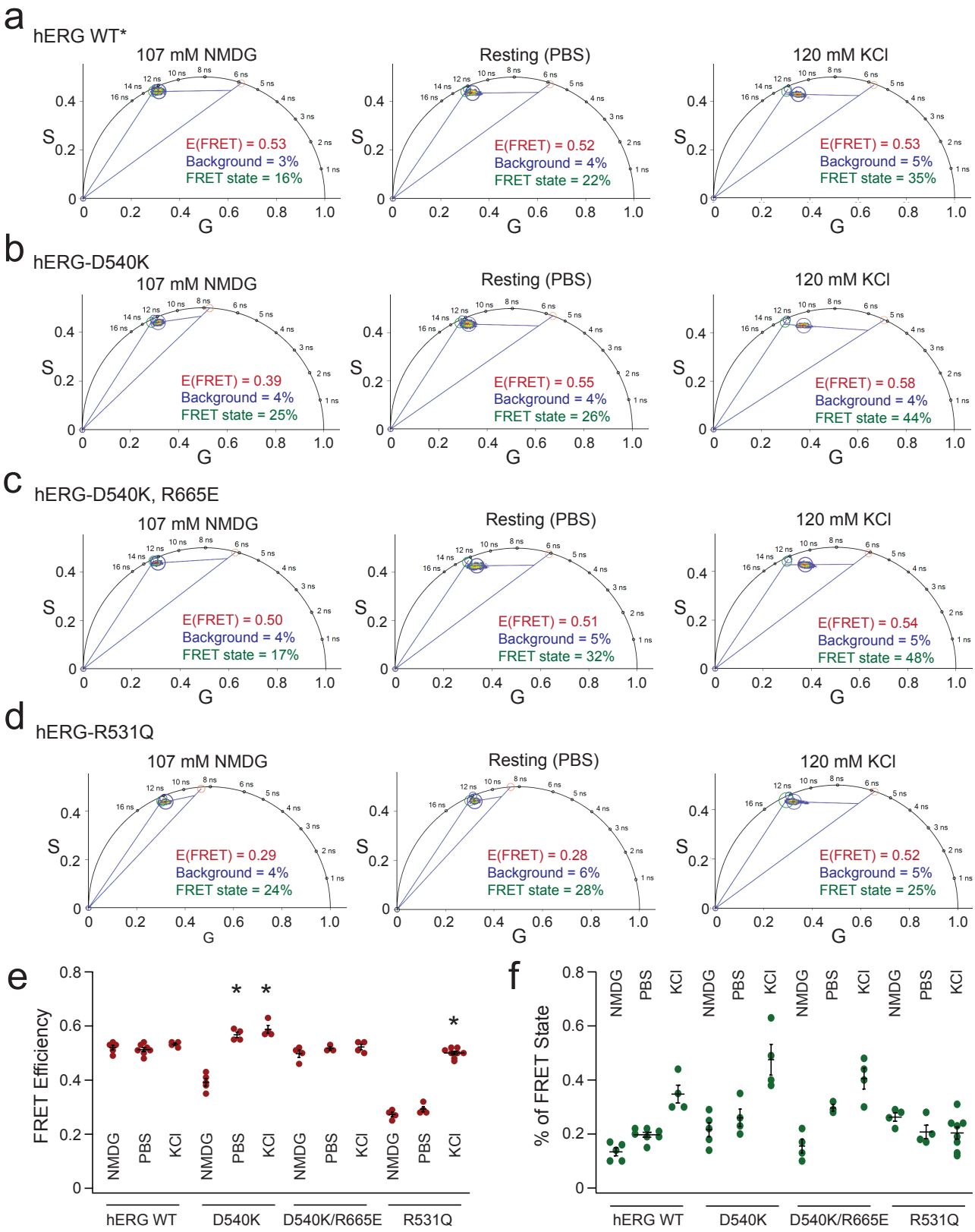

Fig. 4. Phasor linear unmixing analysis of the FLIM-tmFRET in hERG channels under different voltage conditions.

a-d. Representative analysis of the L523/G439 tmFRET from individual cells for hERG WT*, hERG-D540K, hERG-D540K/R665E and hERG-R531Q channels under various conditions.
e-f. Summary data of the estimated FRET efficiencies and percentage of the FRET state using linear unmixing of the phasor approach. Data shown are presented as mean ± s.e.m., n = 3 – 8, *p < 0.05.

Detecting intermediate voltage sensor states in hERG channels containing S4 mutations

Using the same FLIM-tmFRET L523/G439 pair in hERG, we tested the impact of the LQTS mutation R531Q on voltage sensor conformations (Fig. 4d). Our phasor analysis revealed at least two different FRET efficiencies: a lower one around 0.25 and a higher one similar to those observed in tmFRET experiments using the WT* or with combined D540K/R665E mutations (Fig. 4 d-e). There was a notable prevalence of voltage sensors in the FRET state, even under more hyperpolarized voltages, with no increase upon membrane voltage elevation in our experimental conditions. Given the pronounced rightward shift in the G-V curve of hERG-R531Q channels, a more depolarized voltage (> +60 mV) might be necessary to observe an increase in the percentage of the FRET state. These findings suggest the existence of an intermediate conformation of the S4 voltage sensor induced by the R531Q mutation. At hyperpolarized voltages, the voltage sensor primarily transitions between the down and intermediate states relatively frequently (Fig. 4e, percentage of FRET state = ~20%), while at more depolarized voltages, it can ascend to the up state. The measured donor-acceptor distance of 19 Å converted by the intermediate FRET efficiency (in comparison to 13 Å for the high FRET state) aligns with the intermediate conformation of VSD predicted by the cryo-EM structure of Eag channels[11].

We also tested the presence of intermediate FRET states in hERG channels containing the R528Q mutation using the same tmFRET pair. As mentioned earlier, R528Q mutation induced biphasic channel activation, yet did not alter the G-V relationship compared to WT hERG channels (Supplementary Fig. 1d). Similar to the tmFRET results of R531Q mutant channels, phasor analysis revealed the presence of at least two different FRET efficiencies: a lower one around 0.35 (a converted distance of 16.6 Å) and a higher one around 0.5 (Fig. 5), suggesting that the intermediate state of S4 with R528Q likely resides in a slightly more elevated position compared to that of R531Q (Fig. 5e). Notably, unlike the R531Q mutant channels (as depicted in Fig. 4f compared with Fig. 5d), membrane depolarization increased the proportion of FRET states for R528Q-containing channels, indicating a higher probability of the S4 helix in the intermediate or the up states. This observation contrasts with the behavior observed in R531Q mutants and can be attributed to the more leftward G-V relationship observed in R528Q channels (Supplementary Fig. 1). Additionally, we conducted parallel experiments on hERG channels containing the LQTS-related R534Q mutation (Supplementary Fig. 3). These

experiments exhibited only a single high FRET state, distinct from the R528Q but similar to the WT* channels, indicating an absence of S4 intermediate states (Supplementary Fig. 3). Together, our findings suggest that mutations affecting critical voltage-sensing arginines in hERG channels give rise to intermediate conformational states of the S4 helix. These intermediate states offer functional insights into interpreting the intermediate structures initially identified in Eag channels[11] and those proposed in other VGICs[12]. They suggest a novel mechanism through which LQTS-associated mutations can influence the gating of hERG channels and voltage sensing in cardiac tissue.

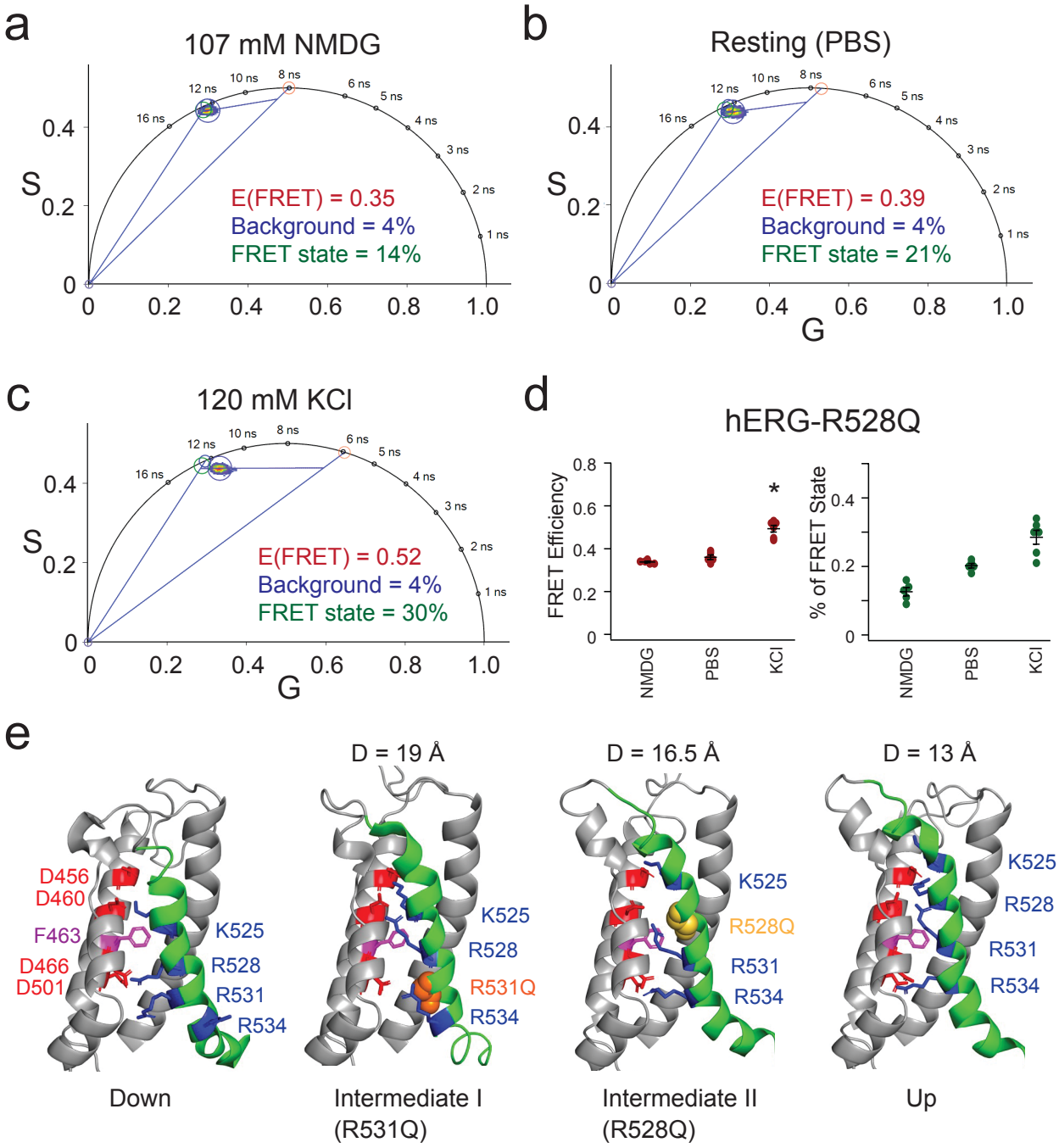

Fig. 5. R528Q, similar to R531Q, revealed distinct intermediate states, analyzed using phasor plots.
a-c. Representative analysis of the L523/G439 tmFRET from individual cells, when R528Q was included.
d. Summary data of the estimated FRET efficiencies and percentage of the FRET state using linear unmixing of the phasor approach. Data shown are presented as mean ± s.e.m., n = 5 – 6, *p < 0.05.
e. Structural cartoons illustrating the intermediate state I and II introduced by R531Q and R528Q, respectively. Presumably, negatively charged residues within the S3 and S4 helices participate to stabilize the intermediate states. Distances indicated are based on the tmFRET measurements between the L523/G439 FRET pair.

DISCUSSION

Currently, there is a lack of effective methods for probing the conformational dynamics and heterogeneity of membrane proteins within living cells. While single-molecule FRET techniques offer substantial advantage in dissecting protein conformational states, their application in studying rapid and voltage-dependent events within native membranes remains challenging. Similarly, obstacles persist in employing methods such as cryo-EM and double electron-electron resonance (DEER), both of which routinely require cryogenic sample freezing. Fluorescence lifetime decays, similar to DEER decays, contain information that can be analyzed through multi-exponential functions, potentially unraveling distinct populations of fluorophores. Notably, these fluorescence lifetime techniques can be executed within native membranes and integrated with functional approaches like patch clamp. Leveraging a combination of genetic code expansion, click chemistry, frequency-domain phasor FLIM, and tmFRET, we engineered a live-cell system to measure intermediate states of ion channel voltage sensors in a relatively high-throughput manner. While the phasor FLIM technique has been used in quantifying multiple states of FRET in the field of cell biology[15], its potential in elucidating the structural biology of proteins is only beginning to be realized. This technique can be broadly applied to study protein dynamics, particularly for membrane proteins.

Building upon our findings, we postulate that neutralization of S4 positive charges gives rise to distinctive intermediate states within the S4 helix voltage sensor (Fig. 5e). Specifically, the negatively charged residues situated within the S2 and S3 helices, known as the extracellular negative cluster (ENC; D456 and D460) and intracellular negative cluster (INC; D466 and D501), play a role in stabilizing these intermediate states. In the case of R531Q mutation, the positively charged side chain of R528 is essential for S4 to interact with the ENC, thereby stabilizing its intermediate state. Conversely, in the instance of the R528Q mutation, S4 depends on the positive charge of the lower R531 to interact with the ENC, thereby resulting in a pronounced upward shift of its intermediate state, compared to that for the R531Q mutation (Fig. 5e). In other words, as the S4 moves upward, an intermediate state occurs when neutralized arginine side chains fail to exchange their ion pair partners

as normal. Interestingly, the contrasting activation patterns observed between R531Q channels (long initial delay and mono-exponential activation) versus R528Q channels (short initial delay but double-exponential activation) may be due to the distinctive functional coupling of the intermediate II S4 conformation to channel opening. Conversely, the intermediate I S4 conformation may lack the capability to lead to pore opening. Furthermore, disrupting the characteristic "RXXR" repeat, which governs arginine positioning, can bifurcate the movement of the voltage sensor into distinct phases. This phenomenon is reminiscent of the biphasic S4 movement observed in the VSD of various other proteins and channels[34-37]. Moreover, the calculated probability of voltage sensors transitioning between distinct FRET states, using linear unmixing phasor analysis, can be calculated using Boltzmann's law to estimate the free energy associated with S4 voltage sensor activation. Future investigations are needed to elucidate the impact of intermediate S4 conformations on the VSD-pore domain (PD) coupling mechanisms underlying hERG channel gating. Additionally, it remains unknown whether a potential 3_{10}-helix to α-helix conversion modulates the intermediate state of voltage sensors[11,38-40].

Our method represents a significant technical advance in the application of FLIM-FRET to structural biology in live-cell imaging experiments and shows promise for future improvement. Enhancing the selectivity of pyrrolysyl-tRNA synthetase for Acd is essential to minimize nonspecific fluorescence. The presence of endogenous genes using the amber codon might interfere with the Acd system, resulting in undesired cytosolic Acd fluorescence in our preparations. Developing an Acd variant that fluoresces exclusively upon incorporation into the protein of interest would help mitigate the nonspecific Acd fluorescence as well. Furthermore, optimizing the linker length between ProK and azido DOTA could bring the transition metal acceptor closer to the protein backbone. This engineering can benefit from more efficient CuAAC click chemistry, requiring lower concentrations of Cu(I) catalyst and exhibiting faster reaction kinetics. Alternatively, engineering copper-free strain-promoted azide-alkyne cycloaddition (SPAAC) reactions could also facilitate labeling of the tmFRET acceptor[41,42]. Such improvements would not only enhance the accuracy of this method but also reduce the cytotoxicity associated with transition metals. Finally, incorporating patch clamp techniques into this method to precisely control membrane voltage could further enhance our ability to correlate channel function with structural states.

MATERIALS AND METHODS

Molecular Cloning

The bioorthogonal labeling of non-canonical amino acids (ncAAs) was achieved by site-specific incorporation using either single or dual codon suppression. This involved using amber (TAG) and ochre (TAA) codons within the channel protein of interest, specifically the hERG1 cDNAs synthesized by VectorBuilder (Chicago, IL). These constructs, featuring a C-terminal eYFP and a CMV promoter, were cloned and amplified using a mammalian gene expression vector. The inclusion of the S620T mutation located in the selectivity filter of the channel in all hERG1 constructs used in this paper served to eliminate the rapid C-type inactivation of the channel[21,43]. Thus, we simplify reference to this hERG1-S620T channel as the WT channel. DNA oligo primers, from Integrated DNA Technologies (IDT, Coralville, IA), were used for the design and synthesis process. Point mutations were induced using either the QuikChange II XL Site-Directed Mutagenesis or Multi Site-Directed Mutagenesis Kit (Agilent Technologies, Cedar Creek, TX). Subsequently, Dpn I restriction enzyme treatment (Agilent Technologies) was applied to digest the methylated parental DNA template of the DNA plasmids, which were then transformed into XL10-Gold competent cells (Agilent Technologies). The introduction of double functional stop codons opal (TGATGA) at the terminal end of YFP ensured successful translation termination. Verification of the introduced stop codons within the mutant plasmid was performed through DNA sequencing provided by either Genewiz (Azenta Life Sciences, Burlington, MA) or Plasmidsaurus (Eugene, OR). Additionally, the DNA concentration was quantified using a Nanodrop OneC spectrophotometer (Life Technologies, Grand Island, NY).

Cell Culture, Transient Transfection and Bioorthogonal Labeling

The tsA-201 cells, a variant of human embryonic kidney (HEK293) cells, were procured from Sigma-Aldrich (St. Louis, MO), authenticated via short tandem repeat (STR) profiling, and then cryopreserved in liquid nitrogen. For initiating new cell cultures, these cells were thawed and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco), within a humidified incubator maintained at 37 °C with 5% CO_2.

Transfection was performed on 70% - 90% confluent tsA-201 cells using the lipofectamine 3000 kit (Invitrogen, Carlsbad, CA, #L30000080), following established protocols[44,45]. For dual stop-codon

suppression, the cells were co-transfected with two plasmids: pAS_4xU6-PylT M15(UUA) FLAG-Mma PylRS (Mma PylT/RS) from Addgene (Watertown, MA, #154774)[25] and the Acd RS1 tRNA/aminoacyl-tRNA synthetase pair (GCE4All Research Center, Oregon State University)[24], along with a third plasmid containing the dual stop-codon mutations required for selective incorporation of two noncanonical amino acids (ncAAs): Acd (the Petersson Lab, University of Pennsylvania) and N-propargyl-L-lysine (ProK) (SiChem, Bremen, Germany), as described previously[25]. To enhance nonsense suppression, a human dominant-negative release factor eRF1 (E55D) (VectorBuilder, Chicago, IL) was co-transfected[46]. The ncAAs were added to the transfection solution: 5 μM Acd and 0.25 mM ProK. ProK was mixed with 1 M HEPES solution in a 1:4 ratio (Sigma-Aldrich, St. Louis, MO) before adding to cell culture. The ProK stock solution (100 mM) were made by dissolving ProK in 0.2 M NaOH water. In the absence of the pAS_4xU6-PylT M15(UUA) FLAG-Mma PylRS (Mma PylT/RS) while in the presence of the Acd incorporation system, we did not observe significant membrane-localized YFP signals (Supplementary Fig. 2e), indicating the Acd RS1 tRNA/aminoacyl-tRNA synthetase pair is specific to the amber stop codon and cannot suppress the ochre stop codon.

At the ProK site, we conducted the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction using 50 μM $CuSO_4$, 250 μM THPTA (Tris(benzyltriazolylmethyl)amine), and 2.5 mM ascorbic acid in DMEM medium[25]. For azido-DOTA labeling (at 100 μM in cell culture), the incubation proceeded for 1-1.5 hours at 37 °C. To produce Cu^{2+}-DOTA, we mixed equal amounts of 100 mM DOTA azide stock and 110 mM $CuSO_4$ stock, allowing them to incubate for five minutes until the mixture transitioned from light to darker blue, indicating Cu^{2+} binding to DOTA. Once bound, DOTA and Cu^{2+} dissociation occurred very slowly due to their picomolar affinity[22]. The azido-mono-amide-DOTA was purchased from Macrocyclics, Inc (Plano, TX).

Fluorescence Microscopy, Fluorescence Lifetime measurement and FLIM-FRET

Fluorescence measurements were conducted using an Olympus IX73 inverted microscope (Olympus America, Waltham, MA), equipped with a 60× U Plan S-Apo 1.2-NA water-immersion objective. Epifluorescence recording was facilitated by wide-field excitation from a pE-800 LED light source (CoolLED, Andover, UK). For choosing cells with strong membrane localized YFP fluorescence via eyepiece of the microscope, YFP was excited using a 500 nm LED line. Acd fluorescence was expected in these cells and was excited using a 365 nm LED line. The imaging solution used for cell visualization was Dulbecco's phosphate-buffered saline (PBS, ThermoFisher), unless specified otherwise. All microscopic experiments were conducted at room temperature.

Spectral measurements of Acd fluorescence used a spectrograph (SpectroPro 2156, 300 groves/mm grating, 500 nm blaze; Teledyne Princeton Instruments, Acton, MA) situated between the microscope output port and an INFINITY3S-1URM CCD camera (Teledyne Lumenera, Ottawa, ON). Acd fluorescence from cells was excited using a 365 nm LED line through a filter cube. The emission filter was removed from the filter cube, allowing only fluorescence in the wavelength range of 423 to 597 nm collected as a spectral image. Spectral images were acquired with exposure times of 500 ms. In addition, spectral measurements of the light absorption of transition metals were made using the Nanodrop OneC spectrophotometer (Life Technologies, Grand Island, NY). Subsequent analysis of the spectral data was conducted using ImageJ/Fiji (National Institutes of Health, USA) and Igor Pro 9 (Wavemetrics, Portland, OR).

Digital frequency-domain fluorescence lifetime imaging was conducted using a Q2 laser scanning confocal system, which was equipped with a FastFLIM data acquisition module (ISS, Inc, Champaign, IL) and two hybrid PMT detectors (Model R10467-40, Hamamatsu USA, Bridgewater, NJ), as previously described[14,22,47]. This confocal FLIM system is integrated with the Olympus IX73 inverted microscope. The fluorescence lifetime measurements encompass both frequency-domain (allowing instantaneous distortion-free phasor plotting) and time-domain decay information. To excite Acd, a 375 nm pulsed diode laser (ISS, Inc) controlled by FastFLIM was used, with the emitted fluorescence captured through a 447/60 nm band-pass emission filter. YFP excitation was achieved using a 488 nm wavelength excitation and collected through a 542/27 nm emission filter. Simultaneous two-color imaging was facilitated by dichroic cubes, with one containing a 50/50 beam splitter and the other equipped with long-pass filters.

For image acquisition, confocal images with a frame size of 256 x 256 pixels were obtained for each experimental condition, using a motorized variable pinhole (set at a size of 100 μm). To determine the phase delays (φ) and modulation ratios (m) of the fluorescence signal in response to an oscillatory stimulus (6 ps per pulse) with a frequency ω = 20 MHz, the VistaVision software (ISS, Inc) associated with the FastFLIM system was used. These values were obtained by performing sine and cosine Fourier transforms of the phase histogram, taking into account the instrument response function (IRF) calibrated with Atto 425 in water (with a lifetime of 3.6 ns, see Supplementary Fig. 2). VistaVision software, equipped with the imaging and FLIM module, was also used for image processing, display, and acquisition. This software allowed for the specification of parameters such as pixel dwell time, region of interest, and resolution. Additionally, it employed a fitting algorithm, phasor linear unmixing

analysis for FLIM-tmFRET analysis. To enhance display and analysis of membrane-localized lifetime species, a median/gaussian smoothing filter and an intensity-based selection of the membrane region were applied. The VistaVision and phasor FLIM provide a significant advantage by effectively differentiating signals originating from background and cytosolic fluorescence outside the membrane region from those within the plasma membrane. This capability is especially important given that our Acd incorporation system tends to generate substantial nonspecific cytosolic fluorescence, which constitutes the primary error source in our experiments. Relying on the selection of cells exhibiting membrane localized YFP fluorescence enabled us to analyze lifetime species of the Acd fluorescence mostly aligned with YFP fluorescence locations (Supplementary Fig. 2). The exact reason for the greater cytosolic Acd fluorescence comparing to other systems that incorporate fluorescent noncanonical amino acids (e.g. L-Anap) via stop-codon suppression is unclear[22,24,29,48], and likely due to the high membrane permeability of Acd and nonspecific amber stop-codon suppression of endogenous genes.

The VistaVision software features the FRET trajectory function to estimate the FRET efficiency, manifesting an averaged distance between donor and acceptor probes. As previously described, key parameters are adjusted to optimize the fitting trajectory through both the donor alone and the donor species due to quenching by FRET acceptors[22]. Regarding the linear unmixing (decomposition) approach of calculating FRET efficiency, the linear fitting process underwent visual inspection to derive four parameters: (1) background contribution in the donor-alone (or low FRET) sample, (2) background contribution in the donor sample in the presence of the acceptor, (3) percentage contribution by the FRET state, and (4) FRET efficiency under theoretical conditions where 100% donors undergo high FRET. Background levels typically remained at or below 5%, consistent with the minimal emission intensity observed in untransfected cells. To determine the percentage contribution by the FRET state, the cursor position was adjusted to cover the central portion of the lifetime distribution. The FRET efficiency of the low FRET state corresponding to the down state of the S4 cannot be accurately measured since the lifetime species typically would not exhibit an oval-shaped distribution and localize very close to those of the donor alone in the phasor plot.

The distances *r* between the Acd and Cu^{2+}-DOTA acceptors were estimated using the convolution of the Förster equation ($E_{max.} = 1 / (1 + (r / R_0)^6)$ with a Gaussian function: Förster convolved Gaussian (FCG) as previously established[9,48]. The FCG adopts a Gaussian distribution with a standard deviation (σ) of 7.5 Å and a full width at half maximum (FWHM) of 17.7 Å (Supplementary Fig. 4). This

assumption was based on the interatomic distance distribution of probes with similar labels, e.g., the L-Anap and Cu^{2+}-TETAC tmFRET pair[9,17,48]. The R_0 value was calculated using the following equation:

$$R_0 = C\sqrt[6]{JQ\eta^{-4}\kappa^2} \qquad (1)$$

where *C* is the scaling factor, *J* is the overlap integral of the Anap emission spectrum and the YFP absorption spectrum, Q is the quantum yield of the Acd (around 0.8), η is the index of refraction, and κ^2 is the orientation factor. η was assumed to be 1.33, and κ^2 was assumed to be 2/3. This calculation generated a R_0 of 13 Å between Acd and Cu^{2+}-DOTA and 12 Å between Acd and Co^{2+}-DOTA. Our FRET distance calculation aimed to provide a quantitative description of conformational heterogeneity rather than determining the precise scale of S4 movement.

Patch-Clamp Electrophysiology

Whole-cell patch-clamp recordings were conducted at a temperature range of 22 - 24 °C using an EPC10 patch-clamp amplifier and PATCHMASTER (HEKA) software. Borosilicate patch electrodes, with an initial pipette resistance of approximately 3 - 5 MΩ, were fabricated using a P1000 micropipette puller from Sutter Instrument (Sutter, Novato, CA). Current signals were low-pass filtered and sampled at a frequency of 10 kHz. Series resistance was mostly under 10 MΩ and compensated at ~ 40-70%. Patch clamp was performed using a Sutter MP-225A motorized micromanipulator (Sutter, Novato, CA).

To record hERG currents from tsA-201 cells, the internal pipette solution was prepared with the following composition (in mM): 10 NaCl, 130 KCl, 3 $MgCl_2$, 10 HEPES, 0.5 EGTA, 1 MgATP, 0.3 Na_3GTP, and pH 7.2 adjusted with KOH. The external solution used was Ringer's solution with the following composition (in mM): 160 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 8 D-Glucose, and pH 7.4 adjusted with NaOH. 10 mM TEA was added to the external solution to block endogenous potassium currents of tsA cells, while having minor effect on the hERG currents[20]. For hyperpolarizing and depolarizing solutions, 107 mM impermeant cation NMDG (N-methyl-D-glucamine) and 120 mM KCl replaced NaCl in the Ringer's solution, respectively[31].

The conductance-voltage (G-V) relationships were determined by analyzing the instantaneous tail currents subsequent to voltage pulse steps. Subtraction of leak tail currents was performed, and the resulting currents were normalized to the maximum tail current amplitude (G/G_{max}). The relative

conductance was then plotted against the voltage of the main pulse and fitted using the Boltzmann equation:

$$G/G_{max}=1/(1 + exp[(V_{1/2} - V)/Vs]) \quad (2)$$

where V is the membrane potential, $V_{1/2}$ is the potential for half-maximal activation, and Vs is the slope factor.

Statistics and Reproducibility

Statistical significance was assessed based on data parameters presented as the mean ± s.e.m. of n independent cells. The two-sided Student's t-test was used for comparing two groups, while a one-way ANOVA followed by Tukey's post hoc test was employed for pairwise comparisons involving more than two data groups. Statistical significance was denoted as $*p < 0.05$ and $**p < 0.01$, respectively.

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AUTHOR CONTRIBUTIONS

G.D. conceived and designed the research. G.D. performed fluorescence imaging experiments. L.J.H. and G.D. designed and performed patch-clamp recordings. E.N.L. and G.D. designed and performed molecular cloning of hERG constructs. G.D. analyzed data and wrote the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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