Biophysical Investigation of Short QT Syndrome-associated Mutation in Voltage-gated Potassium Channels $I_{Ks}$ and KCNQ1

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Leigh Plant, Advisor

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Master of Science
in
Biochemistry

by
Juhee Park

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Last but not least, I would like to thank the Division of Science and the Provost’s Office for the generous summer fellowships and the Brandeis Alumni and Friends scholarship for funding this research.
ABSTRACT

Biophysical Investigation of Short QT Syndrome-associated Mutation in Voltage-gated Potassium Channels $I_{KS}$ and KCNQ1

A thesis presented to the Department of Biochemistry
Graduate School of Arts and Sciences
Brandeis University
Waltham, Massachusetts
By Juhee Park

Short QT Syndrome (SQTS) is a potentially fatal cardiac arrhythmia, characterized by a reduced QT interval in the electrocardiogram. A gain-of-function mutation, V307L, observed in the voltage-gated potassium channel KCNQ1 gene in a SQTS patient has implicated this disease as a cardiac channelopathy. In the heart, KCNQ1 (Q1) subunits assemble with accessory subunits, KCNE1 (E1), to produce the $I_{KS}$ channel, which passes slow outwardly rectifying potassium current that contributes to the cardiac action potential repolarization. To understand the effect of the V307L mutation on the biophysical properties of $I_{KS}$ and Q1 channels, we used whole-cell patch clamp to investigate the channel gating and total internal reflection fluorescence (TIRF) microscopy to visualize the channel expression at the membrane. Electrophysiological studies indicated that the V307L mutation makes the transition from closed to open state more favorable for $I_{KS}$ channels, but not for Q1 channels, by shifting the half-maximal activation voltage and accelerating the activation kinetics. TIRF microscopy illustrated that the V307L mutation decreased the $I_{KS}$ (but not Q1) channel density. In conclusion, we propose two testable mechanisms for E1-dependent gain-of-function phenotype associated with the V307L mutation. The V307L mutation in the pore of Q1 may introduce a partially open state or decrease the energetic barrier to the voltage sensing domain movement upon depolarization of the membrane. These models illustrate how the V307L mutation may result in increased K+ efflux associated with expedited cardiac action potential repolarization and highlight the significance of the 307 site in $I_{KS}$ and Q1 gating.
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**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>E1</td>
<td>KCNE1</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>G</td>
<td>Conductance</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>i</td>
<td>Single channel conductance</td>
</tr>
<tr>
<td>I</td>
<td>Current (macroscopic)</td>
</tr>
<tr>
<td>I_{Ks}</td>
<td>( I_{K_{slow}} ) or outwardly rectifying K^+ current</td>
</tr>
<tr>
<td>Kv channel</td>
<td>Voltage-gated potassium channel</td>
</tr>
<tr>
<td>LQTS</td>
<td>Long QT syndrome</td>
</tr>
<tr>
<td>Q1</td>
<td>KCNQ1</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>SQTS</td>
<td>Short QT syndrome</td>
</tr>
<tr>
<td>SDF</td>
<td>Site-directed fluorimetry</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total Internal Reflection Fluorescence</td>
</tr>
<tr>
<td>TFP</td>
<td>Teal fluorescent protein</td>
</tr>
<tr>
<td>V_{1/2}</td>
<td>Half-maximal activation voltage</td>
</tr>
<tr>
<td>VSD</td>
<td>Voltage sensing domain</td>
</tr>
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</table>
1. Introduction

1.1 Ion channels

Ion channels facilitate the movement of aqueous ions across the lipid bilayer and down an electrochemical gradient near the rate of diffusion [1]. These proteins contribute to basic cellular processes, such as ion homeostasis to protect the cells from exploding from osmotic pressure as well as the fast signal transduction required for sensory perception. The last century of ion channel research has immensely progressed the biophysical and structural understanding of these membrane proteins, which may be extended to understand channelopathies that arise due to an inborn error in channel function or regulation.

As a class of ion channels, voltage-gated channels respond to voltage changes in the membrane potential and gate through conformation changes (Fig. 1). Upon depolarization, a Kv channel in the closed conformation transitions to the open conformation (activation), allowing ions to move across the membrane in a tightly regulated manner (Fig. 1). The open channel may transition to an inactivated state in a voltage-independent manner (inactivation) or back to the closed state (deactivation) upon hyperpolarization, completing the gating cycle.
Fig. 1.1 Kv channel gating is mediated by conformational changes. – adapted [2] A scheme of a Kv channel (Kv 1.1) gating cycle is shown. A Kv channel transitions from the closed to the open state (activation) as the charged voltage sensing domain (VSD) (in black) moves in response to membrane depolarization (indicated by +V) and opens the pore. K⁺ ions can only diffuse across the membrane in the open state. In a voltage independent manner, a Kv channel may transition into an inactivated state. The “Ball and Chain” model of N-type inactivation is shown where the N-terminus forms a ball that blocks the pore of the channel, inhibiting ion movement. After a certain time, the channel recovers from the inactivated state and transitions into the open state. Hyperpolarization of the membrane potential results in a transition from the open to closed state (deactivation) as the VSD returns to the original resting state.

Fig. 1.2 Ion channels and transporters underlay cardiac excitability. – adapted [3] a. A cartoon of key ion channels and transporters expressed in cardiac myocytes is shown. K⁺ channels (green) mediate K⁺ efflux from the cell; Na⁺ channels (purple) and Ca²⁺ channels (yellow) mediate Na⁺ and Ca²⁺ influx, respectively. The Na⁺/Ca²⁺ transporter (red) transports three Na⁺ ions for each Ca²⁺ ion across the membrane. b. A ventricular action potential (in black) is the sum of the underlying ionic currents shown. The name of the current is written to the left of the current traces while the gene encoding for the ion channel or transporter protein is written out to the right. A black box highlights the Iₖs current and the associated KCNQ1 and KCNE1 genes.
1.2 Contribution of $I_{KS}$ to the cardiac action potential

The cardiac action potential is composed of currents from various ion channels expressed in cardiac myocytes (Fig. 1.2). The cardiac action potential commences with fast inward $Na^+$ and $Ca^{2+}$ current which depolarizes the membrane potential. In response, various types of voltage gated potassium channels activate, and the $K^+$ efflux allows the cell to return to the resting membrane potential (repolarization). In particular, a type of slow outwardly rectifying $K^+$ current or $I_{Ks_{low}}$ ($I_{KS}$) current is passed by a distinct Kv channel, the $I_{KS}$ channel complex.

Four Kv7.1 (KCNQ1 or Q1) $\alpha$ subunits and two $\beta$ subunits KCNE1 (E1) assemble to form the $I_{KS}$ channel, a new Kv channel with different biophysical properties [4, 5]. Kv channels possess a tetrameric symmetry, where four $\alpha$ subunits come together to produce the pore of the ion conducting channel [6]. Each $\alpha$ subunit is composed of six transmembrane segments (S1-S6) where the S1-S4 form a voltage sensing domain (VSD) and S5-S6 form a pore domain (Fig. 1.2) [7]. The positive charges on the S3 and S4 of the VSD move upward in response to depolarization of the lipid membrane. The voltage sensor movement is coupled to the opening of the pore, which allows the $K^+$ ions to diffuse down the electrochemical gradient [8]. While $\alpha$ subunits alone can form a functional channel, auxiliary $\beta$ subunits are often found in association. The $\beta$ subunits may modify the channel’s biophysical properties for a more fine-tuned regulation in different tissues or microenvironments.

The addition of the E1 subunit alters the biophysical properties of the Q1 channel (Fig.1.3). The presence of E1 subunits result in an increase in surface expression, hyperpolarization of the voltage-dependence of activation, slowing of gating transition kinetics,
increase in unitary conductance, and alteration of ion selectivity and pharmacology [4, 7, 9-13].

The change of activation and deactivation kinetics as well as increase in current amplitude due to activation at more negative membrane potentials and increased conductance can be seen in $I_{KS}$ current recordings (Fig. 1.3b)

As previously discussed, $I_{KS}$ channels play a crucial role in cardiac action potential repolarization by restoring the resting membrane potential (Fig. 1.2b). Beyond the heart, $Q1$ and $I_{KS}$ are found in other tissues of the body like the epithelial cells of the ear and the gastrointestinal tract and play an important role in salt and water transport [14]. In certain diseases like Jervell and Lange-Nielsen Syndrome, inherited mutations in the $KCNQ1$ and $KCNE1$ gene have been shown to cause both cardiac arrhythmia as well as deafness, illustrating the role that $I_{KS}$ play in both tissues [15].

![Image](image_url)

**Fig. 1.3 E1 alters the biophysical properties of Q1-WT channels.** – adapted [5] a. $K^+$ current, recorded with whole-cell patch clamp in CHO cells expressing Q1 alone and Q1 and E1 ($I_{KS}$). Inset of the tail current shows the magnification of the “hook,” which is thought to show recovery from inactivation for Q1. b. The normalized conductance-voltage relationship for Q1, $I_{KS}$ and fluorescent protein tagged versions of each are shown. The addition of the fluorescent tags to $\alpha$ and $\beta$ subunits does not alter the conductance-voltage relationship and other biophysical properties for Q1 and $I_{KS}$. 
1.3 Short QT syndrome

Short QT syndrome (SQTS) is a less recognized cardiac disease compared to the Long QT syndrome (LQTS), both of which are characterized by a change in the length of the QT interval of an electrocardiogram (ECG) (Fig. 1.4a, b). Since the first clinical report of idiopathic short QT interval in association with sudden cardiac death in 2000, a handful of mutations have been recognized in known cardiac potassium channel genes [16, 17]. These inborn errors illustrate that Short QT syndrome may file under the category of cardiac channelopathy. In particular, SQTS2 has been connected to a gain-of-function Iks phenotype due to a mutation in the KCNQ1 gene (Fig. 1.4c) [18]. In fact, the KCNQ1 gene was historically known as KvLQT1 as earlier studies illustrated that a high number of mutations were associated with sudden cardiac arrests resulting from the most common inherited cardiac arrhythmia, Long QT syndrome [19].

A patient is diagnosed with SQTS, when their QTc (corrected QT) interval is equivalent to or less than 300 ms. This syndrome is associated with cardiovascular risks, including syncope, atrial fibrillation and sudden cardiac death [20]. In a recent long-term ECG study of 1.7 million people, 1 out of 37,000 were found to have a QTc below or equal to 350 ms, and those people were found to be three times more likely to die of cardiac arrest in the ten years following the initial ECG recording [20]. This data suggests that the severe malignancy of the disease may contribute to the small number of SQTS cases reported.
Fig. 1.4 $I_{KS}$-associated cardiac arrhythmia: Short QT syndrome 2 – adapted [21] a. Schematic electrocardiogram (ECG) from a patient with SQTS (in pink) and without the disease (in black). b. Schematic ventricular action potential from a patient with SQTS (in pink) and without the disease (in black). c. Schematic representation of effect of SQTS-associated (gain of function) V307L mutation is shown on $I_{KS}$ current (in pink) compared to wild-type (in black).

Since the detection of the V307L mutation in the $KCNQ1$ gene of a SQTS patient in 2004, several groups have attempted to characterize the effect of this mutation on the biophysical properties of the $I_{KS}$ channel [18]. Based on the electrophysiology data collected from Cos-7 cells, the initial study of the V307L mutation in $I_{KS}$ channels observed accelerated activation kinetics and a hyperpolarization of activation [18]. A different study, conducted in CHO-K1 cells a few years later, reported that they still observed a hyperpolarized activation profile but not the change in activation kinetics, rather a change in deactivation kinetics [22]. To make matters even more confusing, the latest study of $I_{KS}$-V307L channels in CHO-K1 cells reported faster activation kinetics and a previously unseen amplification of $I_{KS}$-V307L current, which could imply an increase in channel density at the membrane [23].
A structural model of the Q1-tetramer with E1 highlights the possible location of the V307L mutation and its putative role in gating. The Q1 tetramer structure, modeled after the crystal structure of another Kv channel (Kv 1.2) illustrates how an E1 subunit may fit in near the pore and between the VSDs [24]. V307 (magenta) is located on the pore helix between the S5 and S6 of the pore domain (yellow), just above the selectivity filter through which the K⁺ ions traverse (Fig. 1.5) [24, 25]. V307 is one of the putative residues that may directly interact with E1 [26]. It is also important to note that the V307L mutation was initially studied as one of the pore residues that may be critical for Q1 inactivation a few years before the discovery in a patient with a SQTS [27]. The proximity of the V307 site to the ion-conducting pore and VSDs emphasize the possible significance of this site in Q1 and Iₖₛ gating as well as its association to Short QT syndrome 2.

**Fig. 1.5 A structural model of KCNQ1 tetramer with KCNE1 highlights the significance of V307.** – adapted [26] A structural model of Q1 tetramer (based on Kv 1.2) with E1 (in cyan) is shown in the closed state: a. top view and b. side view. The VSD S1-S4 is colored in orange, S4-S5 linker in red and the pore domain S5-S6 in yellow. The putative interaction sites for E1 interaction are highlighted in magenta including V307, which is labeled in red. The green highlights Phenylalanine residues, F127 and F130, which are important for modification by β subunit other than E1.
1.4 Aims of this thesis

Despite the decade of research on SQTS2-associated V307L mutation in \( I_{\text{Ks}} \) channels, the measurements of different parameters as well as inequivalent expression systems and electrophysiology techniques under a range of conditions (i.e., temperature) have convoluted our understanding of the biophysical effect of the mutation. To answer the relevant questions required to understand the mutation’s effect on \( I_{\text{Ks}} \) gating, we hope to take advantage of electrophysiology techniques to characterize the biophysical parameters of the \( I_{\text{Ks}} \)-V307L and Q1-V307L current. Whole-cell patch clamp allows the entire population of voltage-sensitive channels at the plasma membrane to be manipulated electrically. A voltage protocol may be used to study different aspects of the voltage-gated channel gating by hyperpolarizing or depolarizing the membrane potential to evoke current response.

To take a step further, we hope to use Total Internal Reflection Fluorescence (TIRF) microscopy to visualize single particles of wild-type and mutant \( I_{\text{Ks}} \) and Q1 channels at the membrane to observe if the V307L mutation alters expression or channel complex assembly [5]. By combining the electrophysiological studies and microscopy of wild-type and mutant \( I_{\text{Ks}} \) and Q1, we hope to propose a comprehensive mechanism on how the V307L mutation may alter \( I_{\text{Ks}} \) and Q1 gating. These models may be used to understand how the mutation may correlate with the Short QT syndrome phenotype.
2. Materials and Methods

2.1 Reagents

**Molecular Biology**: All restriction enzymes and Gibson Assembly kit® were purchased from New England Biolabs. The gBlock Gene Fragments were purchased from Integrated DNA Technologies. All molecular biology was conducted in an in-house vector, pMAX unless specified otherwise.

**Cell culture and expression**: CHO-K1 cells were purchased from American Type Culture Collection. Tissue culture media, serum, transfection reagents were purchased from Life Technologies.

2.2 Molecular Biology

Gibson Assembly was used to introduce the V307L mutation into the human KCNQ1 gene (NCBI ACC# NM_000218) in pRAT, an in-house vector [28]. The Gibson Assembly technique was used to assemble gBlocks, commercially produced double-stranded DNA fragments, with the KCNQ1 gene backbone that was cut with restriction enzymes Afe1 and PshA1 [29]. The gBlock sequences used for the mutagenesis are shown (Fig. 2.1). Q1 gBlock 1 and 2 were used to introduce the V307L mutation into the Q1-WT to produce Q1-V307L. Restriction enzyme digest and ligation were used to insert tagRFP into the c terminus of Q1-V307L to produce Q1-V307L-RFP.
In summary, previously constructed plasmids Q1-WT, Q1-RFP, E1 (ACC#NP_00210.2), E1-TFP and newly constructed Q1-V307L and Q1-V307L-RFP plasmids were used [5].

**Fig. 2.1 Gibson Assembly was used to make the Q1-V307L construct.** a. The DNA sequence of the gBlocks used to introduce the V307L mutation into the human KCNQ1 gene are shown. The underlined regions indicate either the overlap between the gBlocks or with the KCNQ1 gene. b. The linear alignment of the gBlocks 1 and 2 with the human KCNQ1 gene in the in-house pRAT vector. The overlaps with the Q1, restriction enzyme sites and the loci of the mutation are shown.
2.3 Cell Culture and Expression of plasmids

CHO-K1 (Chinese Hamster Ovary or CHO) cells were maintained in F12K medium with 10% FBS and 1% penicillin and streptomycin, volume/volume. To express Q1 or $I_{\text{ks}}$ channel proteins in CHO cells, plasmids carrying Q1 and E1 variants were transfected at concentration of 1 µg per 100 µL of OptiMEM with 2 µL transfection reagent, Lipofectamine 2000. As a transfection marker, 0.3µg of plasmid containing enhanced Green Fluorescent Protein (eGFP) was co-transfected for each transfection unless the Q1 or E1 variant was fused with a fluorescent tag. Exact plasmid and Lipofectamine mixtures for whole-cell patch-clamp experiments and TIRFM experiments are shown (Tables 2.1, 2.2).

Table 2.1 Transfection mixture for whole-cell patch-clamp experiments. The table below shows the amount of DNA for α and β subunits for each channel type and co-transfection marker eGFP as well as the amount of the transfection reagent, Lipofectamine 2000.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Amount of DNA for relevant α and β subunits</th>
<th>Co-transfection marker</th>
<th>Lipofectamine2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>IKs-WT</td>
<td>1 µg KCNQ1-WT + 1 µg KCNE1</td>
<td>0.3 µg eGFP</td>
<td>4 µL</td>
</tr>
<tr>
<td>IKs-V307L</td>
<td>1 µg KCNQ1-V307L + 1 µg KCNE1</td>
<td>0.3 µg eGFP</td>
<td>4 µL</td>
</tr>
<tr>
<td>Q1-WT</td>
<td>1 µg KCNQ1-WT</td>
<td>0.3 µg eGFP</td>
<td>2 µL</td>
</tr>
<tr>
<td>Q1-V307L</td>
<td>1 µg KCNQ1-V307L</td>
<td>0.3 µg eGFP</td>
<td>2 µL</td>
</tr>
</tbody>
</table>

Table 2.2 Transfection mixture for TIRF-M experiments. The table below shows the amount of DNA for α and β subunits for each channel type as well as the amount of the transfection reagent, Lipofectamine 2000.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Amount of DNA for relevant α and β subunits</th>
<th>Lipofectamine 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>IKs-WT</td>
<td>1 µg KCNQ1-WT-RFP + 1 µg KCNE1-TFP</td>
<td>4 µL</td>
</tr>
<tr>
<td>IKs-V307L</td>
<td>1 µg KCNQ1-V307L-RFP + 1 µg KCNE1-TFP</td>
<td>4 µL</td>
</tr>
<tr>
<td>Q1-WT</td>
<td>1 µg KCNQ1-WT-RFP</td>
<td>2 µL</td>
</tr>
<tr>
<td>Q1-V307L</td>
<td>1 µg KCNQ1-V307L-RFP</td>
<td>2 µL</td>
</tr>
</tbody>
</table>
2.4 Electrophysiology

2.4.1 Whole-cell patch clamp [30]

Whole-cell K⁺ currents were recorded with Axopatch 200A/B amplifiers and pCLAMP software (Molecular Devices) from live CHO cells bathed in quasi-physiological solution comprising in mM: 120 NaCl, 4 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 HEPES; pH was adjusted to 7.4 with NaOH. Electrodes were made from borosilicate glass (Harvard Apparatus) and had resistance of 3-6 MΩ when filled with a solution containing, in mM: 130 KCl, 1MgCl₂, 5 EGTA, 5 K2ATP, and 10 HEPES; pH adjusted to 7.4 with KOH. Experiments were performed at room temperature. Currents were evoked with 2 s test pulses between -80 (Appendix) or -100 mV (Results) to +80 mV at 20 mV steps followed by a 2 s tail pulse at -40 mV with holding at -80 mV. The pulse-to-pulse interval was 15 s. The capacitance and series resistance were adjusted each time, using the amplifier. Filtering and sampling frequencies were 5 and 10 KHz.

2.4.2 Analysis of the electrophysiological data

All of the analysis was performed using pCLAMP and OriginLab software.

1. Current density

Isochronal: The current density was calculated by dividing the current amplitude from the end of the 2 s depolarizing test pulse by the membrane capacitance.

Tail: The current density was calculated by dividing the peak current amplitude from the 2 s hyperpolarizing tail pulses at -40 mV by the membrane capacitance.

For Q1-WT, the tail currents were first fit by a single-exponential fit to extrapolate the peak current amplitude before inactivation. Without the extrapolation, the peak tail
current would be at the peak of the “hook,” which would be the max current after inactivation (Fig. 1.1 and 1.3). The resulting peak tail current was used to calculate the current density.

2. Normalized conductance: The peak tail currents were normalized and plotted against membrane voltage to calculate the normalized conductance vs. membrane voltage relationship. This plot was fit by a form of a Boltzmann equation: \( \frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp \left[ -\frac{zF}{RT} (V - V_{1/2}) \right]} \)

3. Activation and deactivation kinetics: Bi-exponential fits of the currents evoked by the depolarizing test pulses were used to measure \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) of activation for \( I_{Ks} \). A mono-exponential fit was used to calculate \( \tau \) of activation for \( Q1 \). The currents evoked by the hyperpolarizing tail pulse at -40 mV were fit by a mono-exponential to calculate the \( \tau \) of deactivation for both \( I_{Ks} \) and \( Q1 \).

4. Percentage current after hyperpolarization: The percentage of the current after hyperpolarization was calculated by dividing the current amplitude at the end of the tail pulse by the peak tail current.

2.5 TIRF Microscopy [5, 30]

Single channel protein complexes at the surface of live CHO cells were identified by TIRF microscopy. RFP, and TFP were excited by 100 mW 561 nm and 40mV 445 nm CellTIRF laser lines. The critical angle for TIRF was adjusted using CellTIRF illuminator (Olympus) and a high-numerical-aperture apochromatic objective (150x, 1.45 NA; Olympus) mounted on an automated fluorescence microscope (IX81; Olympus) controlled by Metamorph software.
For simultaneous illumination of two fluorophores, CellTIRF software (Olympus) was used to adjust the critical angle for each excitation wavelength to generate evanescent waves of equivalent depth of 100 nm. To study RFP and TFP simultaneously, emitted light signals were split by using a 520 nm dichroic mirror mounted in a DualView adapter (Photometrics) and each wavelength was directed to one-half of a back-illuminated EM-CCD (Hammamatsu).

To study the co-localization between two groups of fluorescent particles, Q1-RFP and E1-TFP, unbiased intensity correlation analysis using the Coloc2 plugin was used to obtain the Manders’ coefficient for each cell (ImageJ) [31]. The simultaneously illuminated image collected with DualView was split into two images and aligned using TurboReg plugin to correct for drift (ImageJ).

The particle density was calculated in the following manner. The original TIRF image in greyscale was processed using Threshold (Auto) to produce an image with white background and black particles (ImageJ). A region of interest (ROI) was drawn around the cell parameter to measure the number of particles using the Analyze Particles plugin (ImageJ). Only particles with diameter greater than 0.1 µm were chosen. The representative images from each step for a TIRF image of a CHO cell expressing Q1-RFP alone is shown (Fig. 2.2). The number of particles was divided by the total cell area to calculate the particle density for each CHO cell expressed with either the fluorescently tagged α subunit alone or with the fluorescently tagged β subunit.
Fig. 2.2 Particle density analysis method. Starting from the left, the original TIRF image in greyscale is shown. The middle image is the product of the Threshold step in ImageJ. The last image shows the outline of the chosen particles from the Analyze Particles plugin.

2.6 Statistical Analysis

Kruskal-Wallis ANOVA was used to calculate the statistical significance between the wild-type and mutant measurements (OriginLab). The Kruskal-Wallis ANOVA is a nonparametric test used when analyzing one nominal variable and one measurement variable without the normality assumption. Kruskal-Wallis ANOVA was used over one-way ANOVA as the small N number (3-8) per experiment makes it difficult to determine whether the measurement variable is distributed normally [32].
3. Results

3.1 The V307L mutation alters the activation of $I_{KS}$ channels

Whole-cell patch clamp was performed in CHO cells, expressing $I_{KS}$-WT and $I_{KS}$-V307L channels, to study the effect of the V307L mutation on the biophysical properties of $I_{KS}$ channels. CHO cells express low levels of endogenous $K^+$ channels, which make it a desirable expression system for electrophysiology experiments of $K^+$ channels through transient transfection [5]. The representative whole-cell patch clamp recordings from CHO cells expressing the $I_{KS}$ channels with and without the V307L mutation give an overview of the altered biophysical properties (Fig. 3.1). The depolarizing test pulses were used to study the activation over a range of membrane potential from -100 mV to +80 mV, while the following hyperpolarizing tail pulse was used to study the deactivation after each membrane voltage (Fig. 1.1, 3.1). The initial voltage protocol with test voltages from -80 mV to +80 mV was insufficient in the full characterization of the biophysical effect of the V307L mutation as the $I_{KS}$-V307L started passing current at an unusually hyperpolarized membrane potential compared to $I_{KS}$-WT at -80 mV (Appendix Fig. 5.1).

Further analysis of the current density and kinetics from the patch-clamp current recordings provides an in-depth look at how the V307L mutation may be altering the gating of $I_{KS}$ channels. The analysis over a range of membrane voltages also reveals if the change is voltage dependent. The analysis reports values in the form of mean ± SEM for $n=6$ for $I_{KS}$-WT and $n=7$ for $I_{KS}$-V307L along with p values. Finally, the percentage of current after a
hyperpolarizing tail pulse provides a take-away picture of how the V307L mutation hinders the $I_{Ks}$ channels’ ability to deactivate from the hyperpolarizing -40 mV pulse.

The representative whole-cell patch clamp recordings show that the V307L mutation increases $I_{Ks}$ current production in CHO cells (Fig. 3.1). To further explore this observation, current density was plotted against test pulse voltage (Fig. 3.2a, b). The plasma membrane in a salty solution acts as a capacitor, charging and discharging in forms of capacitance spikes in the beginning and end of recordings (not shown). However, the patch clamp amplifier used for whole-cell patch clamp is able to compensate for the capacitance, which correlates with the overall membrane area and thus can be used as a measure of cell size. Typically, the capacitance per patch of plasma membrane is considered to be 1 µF per cm$^2$ [33]. By dividing the current amplitude by capacitance, the current is normalized per membrane area, giving a current density measurement.
Fig. 3.1 Representative whole-cell patch clamp recordings of CHO cells expressing $I_{Ks}$-WT in a. and $I_{Ks}$-V307L in b. with the corresponding cartoon of α (Q1) and β (E1). The voltage protocol shows the holding potential at -80 mV, followed by a 2s test pulse from -100 mV to +80 mV in 20 mV steps and a 2s tail pulse at -40 mV.
Isochronal current density vs. voltage plot shows a statistically significant increase in $I_{KS}$ current density at voltages of -60 to 0 mV with the addition of the V307L mutation (Fig. 3.2a). The magnification of the $I_{KS}$ current density ranges from minimum of two-fold at -60 mV to with maximum of ten-fold around -20 mV. The current density at -20 mV was $8 \pm 3$ pA/pF for $I_{KS}$-WT and $90 \pm 25$ pA/pF for $I_{KS}$-V307L ($P < 0.01$). However, $I_{KS}$ channels activate very slowly and do not saturate at the end of the 2 s test pulses [5]. This behavior indicates that $I_{KS}$ channels do not reach a thermodynamic equilibrium.

To overcome this challenge, additional current density analysis was performed with peak tail current amplitudes. A step to -40 mV follows after each depolarizing test pulse to evoke the tail current. This transition can be used to capture a thermodynamic snapshot, since the driving force for K$^+$ flux is equivalent for all of the $I_{KS}$ channels under voltage clamp. The sigmoidal shape of the peak tail current density versus membrane voltage plot demonstrates this attempt at achieving a thermodynamic equilibrium (Fig. 3.2b). Further, the peak tail current density vs. voltage analysis illustrates a statistically significant increase in $I_{KS}$ current density from test pulses -100 mV to 0 mV with the addition of the V307L mutation (Fig. 3.2b). The magnification of the current density ranges from minimum of eight-fold at -100 mV to forty-fold at 0 mV. At -20 mV, mean tail current density was $3 \pm 2$ pA/pF for $I_{KS}$-WT and $49 \pm 18$ pA/pF for $I_{KS}$-V307L ($P < 0.01$). Both methods of current density vs. voltage analysis show that current density from CHO cells expressing $I_{KS}$-V307L channels increases by two to forty-fold in a voltage-dependent manner.
Fig. 3.2 Current density versus voltage relationship of $I_{Ks}$-WT and $I_{Ks}$-V307L. a. Isochronal current density measurements taken 2 s from the beginning of test pulses are plotted against the test pulse voltage. b. Peak tail current density measurements are plotted against the test pulse voltage. The data are means ± SEM for $n=7$ for $I_{Ks}$-WT and $n=8$ for $I_{Ks}$-V307L. Statistical significance of the difference between wild-type and mutant was calculated as described in Materials and Methods, * for $P < 0.05$ and ** for $P < 0.01$.

Fig. 3.3 The normalized conductance vs. voltage (G-V) relationship of $I_{Ks}$-WT and $I_{Ks}$-V307L as fit by a Boltzmann relation. a. The normalized conductance of $I_{Ks}$-WT and $I_{Ks}$-V307L are plotted against membrane voltage. b. The $V_{1/2}$ and the slope, biophysical parameters from the Boltzmann fit of the G-V relationship are shown for $I_{Ks}$-WT and $I_{Ks}$-V307L. The data are means ± SEM for $n=7$ for $I_{Ks}$-WT and $n=8$ for $I_{Ks}$-V307L. Statistical significance of the difference between wild-type and mutant was calculated as described in Materials and Methods, * for $P < 0.05$ and ** for $P < 0.01$. 
The V307L mutation shifts the normalized conductance versus voltage (G-V) relationship of $I_{Ks}$ to more hyperpolarized membrane potentials (Fig. 3.3). The normalized G-V relationship illustrates the voltage dependence of steady-state activation. The half-maximal activation voltage ($V_{1/2}$) is a useful measure for comparing the voltage-dependent activation between two channel populations (Materials and Methods). The V307L mutation shifts the $V_{1/2}$ by around -21 mV from $34 \pm 4$ mV for $I_{Ks}$-WT to $13 \pm 6$ mV for $I_{Ks}$-V307L ($P < 0.05$) (Fig. 3.4). This leftward shift of $V_{1/2}$ indicates that less depolarization is required to activate $I_{Ks}$-V307L. The slope demonstrates the change in voltage-dependent relative open probability of the channel (Materials and Methods). The G-V analysis reveals that the slope increases from $21 \pm 1$ mV for $I_{Ks}$-WT to $28 \pm 2$ mV slope for $I_{Ks}$-V307L ($P < 0.05$) (Fig. 3.4b). This increase in the slope may indicate that the V307L mutation promotes $I_{Ks}$ channels to reside in the open conformation longer or more favorably.

The $I_{Ks}$-V307L channels activate significantly faster than the $I_{Ks}$-WT channels at depolarized membrane potentials. The analysis of the activation kinetics shows that the $\tau_{fast, activation}$ of $I_{Ks}$-V307L is about three to five-fold shorter than $I_{Ks}$-WT at membrane voltage of 20 – 80 mV (Fig. 3.4a). At 60 mV, mean $\tau_{fast, activation}$ is $229 \pm 26$ ms for $I_{Ks}$-WT and $73 \pm 12$ ms for $I_{Ks}$-V307L. The characteristic slowness of $I_{Ks}$ activation is altered in the presence of the V307L mutation. In comparison, the introduction of the V307L mutation does not alter the deactivation kinetics of $I_{Ks}$ channels in a statistically significant manner (Fig. 3.4b, Table 3.1).
Fig. 3.4 The voltage dependence of $I_{ks}$ activation and deactivation. a. $\tau_{fast}$ and b. $\tau_{slow}$ of the bi-exponential fit of the activation from currents evoked by the depolarizing test pulses are plotted against membrane voltage. c. $\tau$ of the exponential fit of deactivation from currents evoked by the hyperpolarizing tail pulse is plotted against previous depolarizing membrane voltage. The data are means ± SEM for n=7 for $I_{ks}$-WT and n=8 for $I_{ks}$-V307L. Statistical significance of the difference between wild-type and mutant was calculated described in Materials and Methods, *** for P < 0.005
The V307L mutation hinders the $I_{Ks}$ channels’ ability to deactivate from the hyperpolarizing -40 mV pulse. After the 2 s voltage step at -40 mV, the $I_{Ks}$-V307L channels still pass around 75% of the initial current compared to 25% for $I_{Ks}$-WT (Fig. 3.5). This observation holds true after depolarization to a range of membrane voltages from 20 mV to 80 mV (Fig. 3.5). The residual current after the -40 mV step illustrates that many $I_{Ks}$-V307L channels remain in the open state even after hyperpolarization.

![Graph](image)

**Fig. 3.5 The effect of 2 s hyperpolarizing pulse on $I_{Ks}$-WT and $I_{Ks}$-V307L.** Percentage of current at the end of hyperpolarizing tail pulse (-40 mV) compared to the initial current magnitude highlights the ability of $I_{Ks}$-V307L to still pass current compared to $I_{Ks}$-WT. The data are means ± SEM for n=7 for $I_{Ks}$-WT and n=8 for $I_{Ks}$-V307L. Statistical significance of the difference between wild-type and mutant was calculated as described in Materials and Methods, *** for $P < 0.005$. 

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3.2 The V307L mutation alters Q1 gating in CHO cells

To gain a better understanding of the biophysical effect of the V307L mutation, α subunit Q1 was expressed without E1 in CHO cells. The representative whole-cell patch clamp recordings of Q1-WT and Q1-V307L give an overview of the biophysical properties that may be altered after the introduction of the V307L mutation (Fig. 3.6). The Q1-WT current recordings show the characteristic biophysical behavior compared to $I_{Ks}$-WT: around five to ten fold decrease in isochronal current amplitude, saturation of the isochronal current by the end of 2s test pulses and the appearance of a ‘hook,’ which indicates the recovery from inactivation (Fig. 3.1a, 6a) [5, 34]. The increase in the tail current is thought to represent the current passed by the population of channels that return from the inactivated state to the open state. The ‘hook’ is not observed with Q1-V307L current recordings, which may result from lack of inactivation in the mutant channels (Fig. 3.6) [27].

Similar biophysical analysis: current density, normalized conductance, activation and deactivation kinetics, and current percentage after hyperpolarizing pulse versus membrane voltage were performed to study the impact of the V307L mutation on Q1 gating (Fig. 3.7-3.10). The analysis reports values in the form of mean ± SEM for n= 3 for Q1-WT and n= 5 for Q1-V307L along with p values. Without E1, the V307L mutation’s impact on the aforementioned parameters did not reach statistical significance, other than a two-fold increase in $\tau_{activation}$ at depolarizing membrane potentials: 60 mV and 80 mV (Fig. 3.9a, Table 3.1). This observation indicates that the Q1-V307L activates much slower at these membrane potentials. The biophysical analysis of the currents passed by CHO cells expressing only the α subunit Q1 indicate that the V307L mutation may require E1 to promote activation.
Fig. 3.6 Representative whole-cell patch clamp recordings of CHO cells expressing a. Q1-WT and b. Q1-V307L. The cartoon illustrating the transfected α subunit gene is shown above. The voltage protocol shows the holding potential at -80 mV, followed by a 2s test pulse from -100 mV to +80 mV in 20 mV steps and a 2s tail pulse at -40 mV.

Fig. 3.7 Current density versus voltage relationship of Q1-WT and Q1-V307L. a. Isochronal current density measurements taken 2 s from the beginning of test pulses are plotted against the test pulse voltage. b. Peak tail current density measurements are plotted against the test pulse voltage. The data are means ± SEM for n=3 for Q1-WT and n=7 for Q1-V307L.
**Fig. 3.8** The normalized conductance vs. voltage relationship of Q1-WT and Q1-V307L as fit by a Boltzmann relation. **a.** The normalized conductance of Q1-WT and Q1-V307L are plotted against membrane voltage. **b.** The $V_{1/2}$ and the slope, biophysical parameters from the Boltzmann fit of the G-V relationship are shown for Q1-WT and Q1-V307L. The data are means ± SEM for n=3 for Q1-WT and n=7 for Q1-V307L.

**Fig. 3.9** The voltage dependence of Q1 activation and deactivation. **a.** $\tau_{activation}$ of the exponential fit of the activation from currents evoked by the depolarizing test pulses are plotted against membrane voltage. **b.** $\tau_{deactivation}$ of the exponential fit of the deactivation from currents evoked by the hyperpolarizing tail pulse are plotted against membrane voltage. The data are means ± SEM for n=3 for Q1-WT and n=7 for Q1-V307L. Statistical significance of the difference between wild-type and mutant was calculated as described in Materials and Methods, * for $P < 0.05$. 
Fig. 3.10 The effect of 2 s hyperpolarizing pulse on Q1-WT and Q1-V307L. Percentage of current at the end of hyperpolarizing tail pulse (-40 mV) compared to the initial current magnitude show that both Q1-WT and Q1-V307L deactivate. The data are means ± SEM for n=3 for Q1-WT and n=7 for Q1-V307L.

Table 3.1 Biophysical properties of I$_{Ks}$ and Q1 channels with and without the V307L mutation. The biophysical parameters at representative membrane voltages (-20 mV for current density and 60 mV for activation / deactivation kinetics and % I after hyperpolarization) are summarized (Fig 3.2 - 3.10). The mean ± SEM for each group of wild-type and mutant Q1 and I$_{Ks}$ are shown (N=3-7). Statistical significance of the difference between wild-type and mutant was calculated as described in Materials and Methods, * for P < 0.05, ** for P < 0.01 and *** for P < 0.005.

<table>
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<th>Deactivation</th>
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<td>slope</td>
<td>$\tau_{fast}$</td>
</tr>
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<td>pA/pF</td>
<td>pA/pF</td>
<td>mV</td>
<td>mV</td>
<td>ms</td>
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<tr>
<td>I$_{Ks}$-WT</td>
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<td>2 ± 2 **</td>
<td>34 ± 4 *</td>
<td>21 ± 1 **</td>
<td>229 ± 26 ***</td>
</tr>
<tr>
<td>I$_{Ks}$-V307L</td>
<td>90 ± 26**</td>
<td>49 ± 18 **</td>
<td>13 ± 6 *</td>
<td>28 ± 2 **</td>
<td>73 ± 12 ***</td>
</tr>
<tr>
<td>Q1-WT</td>
<td>23 ± 13</td>
<td>14 ± 8</td>
<td>-19 ± 3</td>
<td>14 ± 2</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>Q1-V307L</td>
<td>39 ± 17</td>
<td>26 ± 12</td>
<td>-27 ± 4</td>
<td>16 ± 1</td>
<td>32 ± 1</td>
</tr>
</tbody>
</table>
3.3 The V307L mutation decreases the $I_{Ks}$ channel density at the membrane.

CHO cells transfected with fluorescently tagged Q1 and E1 subunits were studied at the single molecule level with TIRF microscopy to investigate a possible effect of the V307L mutation on surface expression and the assembly of $I_{Ks}$ complexes. Simultaneous illumination visualized the RFP-tagged Q1 subunits (WT and V307L) and the TFP-tagged E1 subunits from CHO cells transfected with Q1-RFP or Q1-V307L-RFP and E1-TFP (Fig. 3.11, 3.12). The composite image illustrates the co-localization of Q1-RFP and E1-TFP or $I_{Ks}$-FP channel complexes. Wild-type and mutant RFP-tagged Q1 subunits were studied without the TFP-tagged E1 subunit, referred to as groups: Q1-RFP and Q1-V307L-RFP from hereon (Fig. 3.13). Previously published electrophysiological data from the Goldstein group has shown that the addition of the fluorescent tags to $\alpha$ and $\beta$ subunits does not alter the conductance-voltage relationship and other biophysical properties of the associated Q1 and $I_{Ks}$ channels [5]. Thus, the conclusions drawn from the TIRF studies regarding co-localization and channel density at the membrane may serve to explain the electrophysiological findings from the previous section.

The effect of the V307L mutation was analyzed by measuring the co-localization efficiency in the form of Manders’ coefficient and particle density from the TIRF images. The analysis reports values in the form of mean ± SEM for $n=4-6$ cells per group. A mean Manders’ coefficient illustrates the co-localization efficiency between RFP particles and TFP particles (Materials and Methods) [31]. The particle density analysis was used to study the relative ratio of wild-type and mutant $I_{Ks}$ and Q1 populations.
Fig. 3.11 Representative TIRF images of CHO cells transfected with Q1-RFP and E1-TFP. A CHO cell transfected with Q1-RFP and E1-TFP is simultaneously illuminated with the a. RFP laser and b. TFP laser. c. The composite of the two images acquired using split-view is merged to highlight the particles containing IKS-FP channel.

Fig. 3.12 Representative TIRF images of CHO cells transfected with Q1-V307L-RFP and E1-TFP. A CHO cell transfected with Q1-V307L-RFP and E1-TFP is simultaneously illuminated with the a. RFP laser and b. TFP laser. c. The composite of the two images acquired using split-view is merged to highlight the particles containing IKS-V307L-FP channel.

Fig. 3.13 Representative TIRF images of CHO cells transfected with Q1-V307L-RFP or Q1-V307L-RFP. A CHO cell transfected with a. Q1-RFP or b. Q1-V307L-RFP is simultaneously illuminated with the RFP laser.
The V307L mutation does not alter the co-localization efficiency of Q1 and E1 subunits at the cell membrane. The likelihood of finding a TFP particle at the position of a RFP particle on the membrane as measured by the Manders’ coefficient for co-localization is statistically equivalent for Q1-RFP and Q1-V307L-RFP [5, 31]. The Manders’ coefficients for RFP and TFP particle co-localization is 0.76 ± 0.02 for Q1-WT-RFP + E1-TFP group and 0.73 ± 0.02 for Q1-V307L-RFP + E1-TFP group (Fig. 3.14 and Table 3.2). With and without the V307L mutation, around 75 % of RFP particles co-localize with TFP particles. Under the assumption that all RFP particles represent Q1-RFP and all TFP particles represent E1-TFP; the co-localized particles represent I\textsubscript{Ks} channels [5]. This indicates that 75 % of Q1-RFP subunits are found in I\textsubscript{Ks} channels.

The V307L mutation decreases the density of I\textsubscript{Ks} channels at the membrane. The particle density of co-localized particles of I\textsubscript{Ks} channels decrease about 40 % from 10.8 ± 0.1 X 10\textsuperscript{-3} / µm\textsuperscript{2} for wild type to 6.7 ± 0.2 X 10\textsuperscript{-3} / µm\textsuperscript{2} (Fig. 3.15, Table 3.2). However, the particle densities for Q1-RFP alone and E1-TFP alone in the Q1-RFP + E1-TFP groups do not change with statistical significance in the presence of the V307L mutation. The particle density for Q1-RFP alone for the Q1-RFP + E1-TFP group is 3.5 ± 0.2 X 10\textsuperscript{-3} / µm\textsuperscript{2}, compared to 2.4 ± 2.1 X 10\textsuperscript{-3} / µm\textsuperscript{2} for the Q1-V307L-RFP + E1-TFP group (Fig. 3.16, Table 3.2). The above results indicate that only the I\textsubscript{Ks} channel density is altered by the V307L mutation.

The RFP particle analysis in CHO cells transfected with only Q1 confirms the observation that the V307L mutation does not alter the density of Q1-RFP alone. The Q1-RFP density is 6.0 ± 1.3 X 10\textsuperscript{-3} / µm\textsuperscript{2} while the Q1-V307L-RFP density is 4.4 ± 0.3 X 10\textsuperscript{-3} / µm\textsuperscript{2} (Fig. 3.17, Table 3.2). The difference between the wild-type and mutant group does not reach statistical significance (see Statistical Analysis section under Materials and Methods).
**Fig. 3.14** Manders’ coefficient for co-localization of wild-type or mutant Q1-RFP particles with E1-TPF particles. The gray column on the left shows the Manders’ coefficient for cells transfected with Q1-RFP and E1-TPF. The pink column on the right shows the Manders’ coefficient for cells transfected with Q1-V307L-RFP and E1-TPF.

**Fig. 3.15** Particle density in cells transfected with fluorescently tagged α and β subunits. The number of co-localized (RFP and TFP) particles is shown in purple. The density of particles illuminated by the RFP laser but not by the TFP laser is shown in red. The density of particles illuminated by the TFP laser but not by the RFP laser is shown in blue. The left columns with colored lines and clear background were particle density measurements from cells transfected with Q1-RFP and E1-TPF. The right columns with clear lines and colored background were particle measurements from cells transfected with Q1-V307L-RFP and E1-TPF. The data are means ± SEM for n=4 for each group. Statistical significance of the difference between wild-type and mutant was calculated as described in Materials and Methods,* for P < 0.05.
Fig. 3.16 Particle density in cells transfected with fluorescently tagged α subunits. The density of particles illuminated by the RFP laser is shown in red. The left column with colored lines and clear background were particle density measurements from cells transfected with Q1-RFP. The right column with clear lines and colored background were particle measurements from cells transfected with Q1-V307L-RFP. The data are means ± SEM for n=4 for each group.

Table 3.2 Co-localization and particle density of fluorescently tagged wild-type and mutant IκB and Q1 channels. The Manders’ coefficient and particle density for each group are summarized. The mean ± SEM for each group of wild-type and mutant Q1 and IκB are shown (N = 4 for Q1-RFP + E1-TFP and Q1-V307L-RFP + E1-TFP, N=5 for Q1-RFP and N=6 for Q1-V307L-RFP). Statistical significance of the difference between wild-type and mutant was calculated as described in Materials and Methods, * for P < 0.05.

<table>
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<th>Subunits expressed</th>
<th>Manders' coefficient</th>
<th>Particle density $10^{-3}/\mu m^2$</th>
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<tr>
<td></td>
<td></td>
<td>Co-localized</td>
</tr>
<tr>
<td>Q1-RFP + E1-TFP</td>
<td>0.76 ± 0.02</td>
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<tr>
<td>Q1-V307L-RFP + E1-TFP</td>
<td>0.73 ± 0.02</td>
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<tr>
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</table>
4. Discussion

Short QT syndrome 2 and the associated V307L mutation in Q1 provide a unique opportunity to investigate how a relatively conservative mutation may cause carefully regulated ion channels like $I_{Ks}$ to misbehave. Since the initial discovery of the V307L mutation as a product of a single nucleotide polymorphism in a patient with a Short QT Syndrome, several studies have reported a range of effects on $I_{Ks}$ gating [18, 22, 23]. These studies focus heavily on illustrating that the V307L mutation produces an effect which may be corrected by pharmacology. However, the wide range in cell type, temperature, technique and ion concentrations used in the electrophysiology experiments from these studies have resulted in seemingly contradictory results that are difficult to compare. The lack of understanding of the full biophysical effect of the V307L mutation has delayed the production of a cohesive model that may be used to extend our understanding of this cardiac channelopathy [35].

With the combination of whole-cell patch clamp and visualization of the single channel particles at the membrane with TIRF microscopy, we aimed to propose a mechanism that explains the comprehensive effect of the V307L mutation on $I_{Ks}$ and Q1 channel gating. The macroscopic current measured by whole-cell patch clamp is a product of the single channel conductance ($i$), the open probability of the channel ($P_o$) and the number of the channels (N) under clamp. The information regarding i and $P_o$ of the channel may be extrapolated from electrophysiology data in whole cell mode. While single channel studies provide more direct values, gating behavior of single $I_{Ks}$ channels in excised patches have been shown to reflect
whole-cell findings [4]. The particle density analysis from TIRF images provides information on N to illustrate the V307L mutation’s possible effect on expression of the I\textsubscript{KS} and Q1 channels at the membrane. The biophysical characterization of the effect of the V307L mutation on the I\textsubscript{KS} channels by whole-cell patch clamp points toward hyperpolarized and hypersensitive activation. The statistically significant increase in current density, the hyperpolarizing shift in the half-maximal activation voltage and steeper slope of the G-V relationship and shorter $\tau_{fast,activation}$ of I\textsubscript{KS}-V307L illustrate that less electrochemical driving force is required to activate I\textsubscript{KS}-V307L under physiological conditions (Fig. 3.1-3.5, Table 3.1). This gain-of-function phenotype correlates with Short QT syndrome as greater K\textsuperscript{+} efflux will expedite the repolarization and shorten the cardiac action potential and consequently the QT interval.

The electrophysiology results from this thesis reflect a combination of the observations on altered I\textsubscript{KS}-V307L gating from previously published studies. The hyperpolarizing shift of $V_{1/2}$ of around –20 mV and the three-fold acceleration of the activation kinetics as measured by the $\tau_{fast,activation}$ at 60 mV (Fig. 3.3 and 3.4) are within the error of the mean of the published values [18] [22]. The ten-fold increase in isochronal and tail current density measurements at -20 mV are also similar to the published values (Fig. 3.2) [23]. This biophysical analysis illustrates that the V307L mutation makes activation more favorable for I\textsubscript{KS} channels, especially near the resting membrane potential. Finally, the results from this thesis illustrate for the first time that the aforementioned change in gating parameters require the presence of E1.

Without E1, the V307L mutation does not induce a statistically significant change in current density or the G-V relationship of Q1 channels (Fig. 3.7, 3.8, 3.10, Table 3.2). However, the V307L mutation increases $\tau_{deactivation}$ at depolarized membrane voltages and abolishes
inactivation (Fig. 3.6, 3.9, Table 3.2). These additional insights show that the mutation may disrupt Q1 gating in a distinct manner, compared to its effect on the lks channels. This conclusion highlights the importance of the β subunit association in the potency of the V307L mutation for increased K⁺ flux. Additionally, the differential impact on Q1 gating implicates that the 307 site in the pore of the α subunit may serve an important role in the E1 modulation of Q1 gating (Fig. 1.5).

The visualization of the fluorescently tagged Q1 and E1 subunits by TIRF microscopy illustrates a statistically significant decrease in the lks-V307L channel density at the membrane (Fig. 3.15, Table 3.2). This observation can be used to rule out a possible hypothesis that the lks-V307L current density is increased simply due to higher channel expression. A feasible explanation for this phenotype may be that the cell’s quality-control system is able to recognize the lks-V307L channels and degrade the mutant channels, similar to the outcome of F508del Cystic fibrosis transmembrane conductance regulator (CFTR) in Cystic Fibrosis [36]. Experiments to assess the stoichiometry of α and β subunits may be useful in observing if the V307L mutation disrupts the formation of holo-lks complexes [5]. A deviation from the expected 4:1 Q1:E1 ration may indicate that the lks assembly process may be disrupted by the V307L mutation.

Similar to the electrophysiological findings, the V307L mutation has little or no impact on Q1 particle density when E1 is not present. The particle density is statistically equivalent for Q1-RFP and Q1-V307L-RFP (Fig. 3.14, 3.16, Table 3.2). These results indicate that the V307L mutation does not alter the expression of the alpha subunit Q1 alone.
Compared to the published values, both the particle density for $I_{Ks}$ and Q1 channels and the Manders’ coefficient of co-localization are smaller [5]. The $I_{Ks}$ and Q1 channel density from TIRF experiments are around 50 times smaller and the Manders’ coefficients are about 20 % smaller than the published values. These results may be due to the relatively low number of cells analyzed due to the time constraint. While the absolute values of the particle density and the Manders’ coefficients from the thesis may be different from the published literature, the relative ratio between the wild-type and V307L group may still be valid, as equal numbers of cells were analyzed for each group using the same analytic method.

Based on our findings from electrophysiology and TIRF studies, we propose two mechanisms to explain the effect of the V307L mutation on Q1 and $I_{Ks}$ gating. The first model illustrates that the V307L mutation introduces another open state, preceding the regular open state (Fig. 4.1). The disruption in the pore by the V307L mutation may make it favorable for a transition from the closed state to a partial open state ($O_{I_{Ks}}$), which requires less depolarization (as indicated by the small $+v$). The altered biophysical properties of $I_{Ks}$ channels like hyperpolarized $V_{1/2}$ and the steeper slope of the G-V relation, faster activation kinetics and significant amount of residual current following hyperpolarization support this model. Without the E1, the V307L mutation induces a new open state ($O_{Q1}^*$) that has difficulty transitioning to both the closed and the inactivated state (Fig. 4.1). This reflects the similarity in the G-V relationship, slowing of activation kinetics as well as the lack of “hook” for Q1-V307L channels.

A second model proposes that the V307L mutation lowers the energetic barrier of activation by accelerating the VSD movement (Fig. 4.2a). To transition from the closed to the open state, the VSD goes through two steps: the initial movement from the resting to active
state followed by a coupled opening of the pore (Fig. 1.1, 4.2a). Recent studies have shown that the \( I_{Ks} \) channels activate slowly as the addition of the E1 impedes the VSD transition from the resting to the active state (Fig. 4.2a). The V307L mutation may alleviate the slowing effect of E1 by accelerating this transition, allowing the conformation change from the closed to the open state to be more favorable (Fig. 4.2b).

The minimal effect on Q1 activation by the V307L mutation indicates that the coupled pore opening step may not be affected. While it is possible that the V307L mutation may affect the pore opening, this would not be detected by whole-cell patch clamp of \( I_{Ks} \) channels, for which the rate limiting step is the initial VSD movement. However, for Q1 alone, the decrease in the barrier to pore opening step would be reflected as the hyperpolarization of activation. The results from whole-cell patch clamp experiments of Q1 channels indicate that the voltage-dependent Q1-V307L activation is similar to that of Q1-WT (Fig. 3.8). The lack of inactivation for Q1-V07L further supports the decrease of the energy barrier to the VSD transition (Fig. 4.2b). The V307L mutation may make it more favorable for VSD to return to the resting state, preventing the Q1 channels from transitioning from the open to the inactivated state.

The model #1, model #2 or a combination of both models may explain the effect of the V307L mutation on \( I_{Ks} \) and Q1 gating. More electrophysiology experiments may be used to strengthen the validity of the gain-of-function mechanism underlying this fascinating and physiologically relevant mutation. Site-directed fluorimetry (SDF) experiments may be used to analyze the VSD movement of \( I_{Ks}-V307L \) and Q1-V307L channels. SDF correlates the change in fluorescence intensity of a probe with the VSD movement [28]; Zaydman, 2014 #422]. The fluorescent probe (attached to the VSD) experiences a different microenvironment, from the
hydrophobic lipid bilayer to the aqueous extracellular solution, through the transition of the VSD from the resting to the activated state and the pore opening [28]. The results of the SDF experiments with Q1-V307L and I_{KS}-V307L channels may provide additional insight on the validity of the model #2.

Application of known blockers of KCNQ1 and I_{KS} may be used to test model #1 by investigating the possibility of a V307L-induced open state. In fact, a study on determining the binding sites for Chromanol 293b, a well-known KCNQ1 blocker, showed a three-fold decrease in block for Q1-V307L channels [37]. Further, the introduction of the valine to the analogous site (L272V) in the related KCNQ2 channel partially conferred the novel sensitivity to Chromanol 293b. The decrease in Chromanol 293b block on Q1-V307L channels as well as the introduction of sensitivity by exchanging the analogous Leucine with a Valine in the related KCNQ2 channels indicate that the V307L mutation may induce a structural change to produce a distinct open conformation with altered pharmacology.

The ten-fold increase in Chromanol 293B sensitivity in the presence of E1 has been connected to the drug binding more favorably to channels in the open conformation [38]. Following this logic, the application of Chromanol 293b on I_{KS}-V307L may reveal an even higher increase in the drug affinity or altered blocking phenotype as these mutant channels may have another distinct open state as indicated by the first model (Fig. 4.1). The future studies on the application of Chromanol 293b on I_{KS}-V307L channels will provide insight on the therapeutic potential of Chromanol 293b for SQTS2 as well as its potential role in illustrating the role of V307L mutation in altering I_{KS} gating.
Fig. 4.1 Model #1: V307L introduces a new open state for $I_{Ks}$ and Q1. The gating scheme illustrates the introduction of a partial open state ($O_{IKs}^o$) preceding the regular open state ($O_{IKs}$) for $I_{Ks}$-V307L (in red) compared to $I_{Ks}$-WT (in black). The gating scheme for Q1-V307L (in red) shows the new open state $O_{Q1}^*$, which replaces the open state ($O_{Q1}$) of Q1-WT (in black).

Fig. 4.2 Model #2: V307L mutation lowers the energy barrier of VSD movement from resting to active state. 

a. The energetic landscape of the VSD movement to induce activation or the pore opening - Adapted [28]. The increase in the energetic barrier between the resting and the active state by the addition of E1 (in blue) and the decrease by the V307L mutation (in red) are shown. b. The gating scheme illustrates the relatedly easier activation of $I_{Ks}$-V307L (in red) vs. $I_{Ks}$-WT (in black). The lack of inactivation step in the gating of Q1-V307L (in red) is shown, in relation to the gating of Q1-WT (in black).
5. Appendix

Fig. 5.1 The representative recordings and G-V relationship of $I_{Ks}$-WT vs. $I_{Ks}$-V307L for depolarization from -80 mV. The representative recordings starting at -80 mV of CHO cells expressing a. $I_{Ks}$-WT (in black) and b. $I_{Ks}$-V307L (in red). c. The normalized conductance of $I_{Ks}$-WT and $I_{Ks}$-V307L are plotted against membrane voltage. b. The $V_{1/2}$ and the slope, biophysical parameters from the Boltzmann fit of the G-V relationship are shown for $I_{Ks}$-WT and $I_{Ks}$-V307L. The data are means ± SEM for n=5 for $I_{Ks}$-WT and n=6 for $I_{Ks}$-V307L. Statistical significance of the difference between wild-type and mutant was calculated as described in Materials and Methods, ** for P < 0.01.
6. References

29. Gibson, D.G., et al., *Enzymatic assembly of DNA molecules up to several hundred kilobases.* (1548-7105 (Electronic)).