Arrhythmogenesis in Timothy Syndrome is associated with defects in Ca$^{2+}$-dependent inactivation

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Timothy Syndrome (TS) is a multisystem disorder, prominently featuring cardiac action potential prolongation with paroxysms of life-threatening arrhythmias. The underlying defect is a single de novo missense mutation in Ca$_v$1.2 channels, either G406R or G402S. Notably, these mutations are often viewed as equivalent, as they produce comparable defects in voltage-dependent inactivation and cause similar manifestations in patients. Yet, their effects on calcium-dependent inactivation (CDI) have remained uncertain. Here, we find a significant defect in CDI in TS channels, and uncover a remarkable divergence in the underlying mechanism for G406R versus G402S variants. Moreover, expression of these TS channels in cultured adult guinea pig myocytes, combined with a quantitative ventricular myocyte model, reveals a threshold behaviour in the induction of arrhythmias due to TS channel expression, suggesting an important therapeutic principle: a small shift in the complement of mutant versus wild-type channels may confer significant clinical improvement.

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Timothy Syndrome (TS) is a multisystem channelopathy in which patients suffer from long QT syndrome, immune deficiencies and autism. TS presents in two major forms differing primarily in their TS channel expression pattern. In TS type 1, a G406R mutation occurs within the mutually exclusive exon 8a (using the nomenclature of Splawski et al.) splice variant of CaV1.2. This variant is expressed in a wide distribution of tissues, but represents only 20% of CaV1.2 channels in the heart. This explains the multisystem nature of TS type 1, which includes prolonged QT intervals, syndactyly, immune deficits, developmental delays and often autism. However, the lower expression profile of TS type 1 in the heart and brain seems to be more compatible with survival, and thus accounts for the majority of reported cases of TS. In TS type 2, on the other hand, the mutation (either G406R or G402S) occurs within the alternate exon 8, which is expressed in a smaller subset of tissues, but represents the more prominent splice variant in the heart (80% of cardiac CaV1.2). TS type 2 patients are rare, but cases have been found for both the G406R and G402S variants. As a result, these differing expression profiles, TS type 2 patients lack syndactyly while displaying more severe cardiac deficits, with profoundly prolonged QT intervals.

In both variants of TS, the mutations cause a disruption in the function of the CaV1.2 voltage-gated Ca\(^{2+}\) channel. These channels are critical conduits of Ca\(^{2+}\) influx, attenuate Ca\(^{2+}\) influx, and thus reduce F\(_{CDI}\) (Fig. 2b, blue). In this way, channels would exhibit lower CDI due to dissection of Ca\(^{2+}\) regulatory changes caused by each TS mutation. Here, we chose conditions which minimize VDI in CaV1.2, allowing isolated study of CDI. To accomplish this, we co-expressed our human CaV1.2 channels with the \(\beta\_2\) auxiliary subunit, which is known to decrease VDI in Ca\(^{2+}\) channels. In addition, as exons 1 and 8 are known to co-spli, we constructed two naturally expressed human cardiac (exon 1/exon 8) and smooth muscle (exon 1/exon 8a) splice variants harbouring the TS mutations. We could now use whole-cell patch-clamp recordings to measure CDI (Fig. 1b), which can be seen as the faster decay of the Ca\(^{2+}\) current (red) as compared with the Ba\(^{2+}\) current (black) and is quantified as the ratio of Ca\(^{2+}\) current remaining after 300 ms (\(r_{300}\)). Under these conditions, both G402S and G406R channels exhibited a striking reduction of CDI (Fig. 1) as compared with wild-type (WT) CaV1.2 channels. Further, this reduction in CDI was clearly present in either of the physiologically relevant splice backgrounds (Supplementary Fig. 2). We thus demonstrated a strong effect of either TS mutation on CDI in CaV1.2 channels. In fact, even in the context of strong VDI seen in the presence of the \(\beta\_2\) subunit, the effects on CDI are readily apparent in both splice backgrounds (Supplementary Figs 3 and 4) and can be detected in adult myocytes (Supplementary Fig. 5). In all, these data firmly establish a clear effect on CDI by either of the two TS mutations across multiple experimental conditions.

An allosteric model predicts opposing mechanisms of CDI loss. We next turned to the underlying mechanisms of CDI disruption. We began by considering an allosteric mechanism of CDI known to underlie gating of CaV1.2 and CaV1.3 channels. According to this model (Fig. 2a), in the absence of CDI, channel gating is described by a ‘mode I’ scheme (top row), where \(Q_{eff}(V)\) is the voltage-dependent equilibrium constant of the channel voltage sensors, \(L\) governs the voltage-independent final concerted opening step of the channel and is a scaling factor representing the effects of the TS mutations on channel activation (\(a = 1\) for WT channels). Upon opening, Ca\(^{2+}\) enters and binds to CaM (black circles), ultimately driving the channel into the ‘mode Ca’ regime (bottom row). This mode is characterized by a lower open probability that results from a decreased equilibrium constant between the last closed state and the open state. In particular, the magnitude of decrease is specified by factor \(f\). According to this allosteric mechanism, CDI can be decomposed into two components. The first is specified by the metric \(F_{CDI}\), which quantifies the fraction of channels that transition from mode 1 to mode Ca in the steady state. As expected, \(F_{CDI}\) depends upon \(J(Ca^{2+})\). The second component is quantified by \(CDI_{max}\), the CDI that would be observed if all of the channels were to transition from mode 1 to mode Ca. Accordingly, \(CDI_{max}\) reflects the reduction in open probability of mode Ca relative to mode 1. The combination of these two components thereby defines the total CDI that results at steady state, where \(CDI = CDI_{max} \times F_{CDI}\). Given this framework, we could conceptualize the potential effects of the TS mutations. Since these mutations are situated near the intracellular channel activation gates, one might expect changes in the ease of transitions from closed to open states, as represented by the scaling factor \(a\) applied to the final opening step. By representing this scaling factor in terms of the change in energy required for the channel to open (\(\Delta G_\alpha = RT \cdot \log(a)\)), simulations of the model in Fig. 2a suggest that reductions in CDI could actually arise in two distinct ways. First, mutations that make opening more difficult \((a < 1, \Delta G_\alpha > 0)\) would decrease Ca\(^{2+}\) influx, and thus reduce \(F_{CDI}\) (Fig. 2b, blue). In this way, channels would exhibit lower CDI due to

Results

TS mutation effects on calcium-dependent inactivation. Given the ambiguity regarding the effects of the TS mutations on the biologically relevant CDI process, we first undertook careful

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increased occupancy of mode Ca at steady state (case 1). Alternatively, if the channel becomes easier to open \((\alpha > 1, \Delta \Delta G_a < 0)\), then the open probability in mode Ca would also increase, resulting in decreased CDI_{max} (Fig. 2b, green). In this scenario, CDI decreases despite entry into mode Ca, because of an attenuated decrement in open probability upon entering mode Ca from mode 1 (case 2). Thus, either a decrease or an increase of energy required for the final opening step may reduce CDI, but with an important predicted difference. For case 1, the \(F_{\text{CDI}}\) reduction is primarily due to decreased \(\text{Ca}^{2+}\) influx and too little \(\text{Ca}^{2+}\) driving entry into mode Ca. As such, CDI could be restored if sufficient \(\text{Ca}^{2+}\) were available. Case 2, on the other hand, is the result of a reduction in CDI_{max} which cannot be overcome by increasing the \(\text{Ca}^{2+}\) that triggers inactivation.

To explore whether TS mutations might actualize one or both of these model scenarios, we scrutinized mutant TS channels for changes in channel activation. To this end, we utilized single-channel recordings to determine the open probability \((P_o)\) of the channels as a function of voltage (Fig. 3). Here, voltage ramps were applied to single CaV1.2 channel with \(\text{Ba}^{2+}\) as the charge carrier. Initially, gating corresponds to typical CaV1.2 mode 1 behaviour (Fig. 2a)\(^{24}\), while the addition of Bay K 8644 resulted in a substantial increase in mode 2 gating in both TS mutants (Fig. 3b) as expected for CaV1.2 (ref. 26). This increased channel opening permitted clear resolution of the single-channel conductance (dashed grey line; \(g = 0.018 \text{pA mV}^{-1}\)), and significantly aided in determining the number of channels in each patch, required for accurate calculation of \(P_o\). We could now divide the ensemble average current into the fully open current level, to get the \(P_o-V\) relationship. We find that WT and both TS mutant channels share a maximal \(P_o\) of 0.4 at saturating depolarization (Fig. 3d). On the other hand, the \(V_{1/2}\) of each construct is markedly different from the WT. The G402S mutation confers an \(-10\text{-mV}\) depolarizing shift in activation (Fig. 3d, middle; Supplementary Fig. 6), consistent with case 1 of our model \((\Delta \Delta G_a < 0)\). In contrast, the G406R channels exhibit an opposite, \(-10\text{-mV}\), left shift of \(P_o-V\) curves (Fig. 3d, right; Supplementary Fig. 6), fitting with a case 2 profile \((\Delta \Delta G_a > 0)\). This dichotomy of effects raised the intriguing possibility that the two TS mutations in fact elaborate opposing mechanisms of CDI loss, despite superficial similarities in their inactivation defects. Contrasts in the actual underlying mechanism of action might then explain differences in disease progression among patients, and point the way to improved therapies customized to the individual challenges of each scenario.

**Direct evidence for opposing mechanisms of CDI loss.** Though compelling, the contrasting \(P_o-V\) relations for WT and TS channels in themselves do not substantiate the existence of case 1 and/or case 2 mechanisms. Direct tests of such mechanisms could be undertaken if we could control the \(\text{Ca}^{2+}\) signal driving CDI, in a manner independent of variable \(\text{Ca}^{2+}\) fluxing through channels with differing activation gating properties. This

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**Figure 1 | Timothy Syndrome mutations reduce CDI.** (a) Diagram of Timothy Syndrome mutations within human CaV1.2. Exon 8 is expected to be paired with exon 1a in cardiac channels. (b) Exemplar whole-cell current traces in \(\text{Ca}^{2+}\) (red) and \(\text{Ba}^{2+}\) (black). CDI is seen as the faster decay of the \(\text{Ca}^{2+}\) current. Scale bars are 200 pA and refer to the \(\text{Ca}^{2+}\) conductance (dashed grey line; \(g\)). Opening permitted clear resolution of the single-channel carrier. Initially, gating corresponds to typical CaV1.2 mode 1 behaviour. (c) Population data, the fraction of peak current remaining after 300-ms depolarization \((f_{300})\) is plotted for \(\text{Ba}^{2+}\) and \(\text{Ca}^{2+}\) currents. The difference between \(\text{Ca}^{2+}\) and \(\text{Ba}^{2+}\) relations at 30 mV \((f_{300})\) specifies the isolated effect of CDI. Error bars represent ± s.e.m., \(n = 10, 8\) and 12 for WT, G402S and G406R, respectively.
Case 2 mechanism

Channels in mode Ca (dependent on reducing Ca²⁺ concentration). The combination of these two curves defines the total CDI of a channel (black). To this end, we employed Ca²⁺ robust definition of a Ca²⁺ display little inactivation when internal Ca²⁺ relative isolation (Supplementary Fig. 7).

To this end, we utilized our Ca²⁺ capability would allow for direct control over FCDI (a function of Ca²⁺), while leaving CDI max (a function of channel gating) unaltered. To this end, we employed Ca²⁺ photo-uncaging to manipulate the intracellular Ca²⁺ concentration. In addition, we used the monovalent permeant ion Li⁺ as the inward charge carrier through channels, so as to ensure that uncaged Ca²⁺ was the sole source of Ca²⁺. However, this configuration of channel permeation heightens the chance of pore block at the channel selectivity filter by uncaged Ca²⁺ (Supplementary Fig. 7); such blockade would complicate resolution of CDI apart from block. To reduce this effect, we utilized a known pore mutation (E736A) which reduces the selectivity of the channel for Ca²⁺ versus Li⁺ (ref. 29), thus allowing for resolution of CDI in relative isolation (Supplementary Fig. 7).

Thus armed, we undertook Ca²⁺ photo-uncaging of TS channels. DM nitrophen (DMNP-EDTA) provided an appropriate Ca²⁺ cage, supporting near step release of Ca²⁺ upon brief ~1 ms flashes of ultraviolet (UV) excitation (measured by Ca²⁺ imaging with Fluo-4-FF). Combined with patch-clamp electrophysiology, Ca²⁺ uncaging provided a controlled Ca²⁺ input and a clear readout of CDI. As before, CaV 1.2 channels displayed little inactivation when internal Ca²⁺ was kept low (Fig. 4a, top). When Ca²⁺ was uncaged during the depolarizing step, CDI manifested as a prominent decay in current (Fig. 4a, bottom). By measuring this CDI at various Ca²⁺ levels (Fig. 4b), robust definition of a Ca²⁺ response curve for CaV 1.2/CaM could be achieved by plotting the extent of inhibition versus the Ca²⁺ concentration (Fig. 4d). For this curve to be fully reflective of CDI alone, any residual pore block must first be accounted for. To this end, we utilized our Ca²⁺ uncaging protocol on the same channels, but co-expressed with a mutant CaM (CaM E736A) which lacks Ca²⁺ binding (Fig. 4c). In this configuration, channels could not undergo CDI and we could independently measure the remaining pore block of the channel. We found that Ca²⁺ pore block had a Kd of 24 µM, and a Hill coefficient of unity, regardless the type of channel (Fig. 4c, d), an outcome consistent with the original description of the E736A mutation 27,29. We could now divide the residual pore block relation into our uncaging data, thus explicitly resolving the Ca²⁺ response curve for CDI in the CaV 1.2/CaM complex (Fig. 4e).

As expected from whole-cell recordings, CaV 1.2 channels exhibit a large CDI max (0.77), with a Kd of 0.78 µM and a Hill coefficient of 2 (Fig. 4e). Turning to the G402S mutation, case 1 predicts that the FCDI deficit of these channels should be rectified by a sufficiently large Ca²⁺ input. Indeed, G402S channels exhibited pronounced CDI in response to a large Ca²⁺ step (Fig. 4a,b, middle) resulting in a CDI max comparable to WT. This outcome directly establishes a case 1 scenario. G406R, on the other hand, fails to exhibit enhanced CDI, even in the presence of a saturating Ca²⁺ signal (Fig. 4a,b, right). Indeed, response curve analysis of this mutant channel revealed a considerable reduction in CDI max, directly substantiating a case 2 scenario (Fig. 4e, right).

Thus, we have validated two opposing mechanisms underlying TS CDI deficits: G402S causes a decrease in FCDI, while G406R primarily results in a reduction of CDI max.

Implications for pathogenesis. Given the clear contrast between the biophysical mechanisms underlying the two TS mutations, we explored potential differences in pathogenesis. As a first step, we focused on the cardiac effects of TS, utilizing a widely employed ventricular myocyte model, the Luo–Rudy (LRd) model 30. This in silico model, formulated for guinea pig, was here customized to incorporate 1-type channel parameters constrained by our experimental measurements, and to more closely approximate the baseline behaviour of the human heart. In particular, the tail-
current activation curves, obtained for each channel in this study (Supplementary Fig. 6), were used to constrain the simulated L-type channel activation properties as shown in Fig. 5a. Here, the solid lines represent model fits, and circles reproduce experimental data shifted to account for surface charge effects. Additionally, inactivation parameters for both VDI and CDI were adjusted to correspond to data from this study. Finally, we explicitly simulated two pools of L-type channels, to account for the heterozygous nature of TS mutations. Because TS1 and TS2 differ primarily in their expression levels in the heart, we allowed the fraction of TS-affected channels to be adjusted in the model.

With appropriate customizations of the LRd model in hand (see Supplementary Note for details), we looked first at the APs generated with the WT CaV1.2 channels at steady state. Here, in response to 1 Hz pacing, we obtained an APD of 300 ms (Fig. 5b), and a slower timebase displays that the repetitive electrical responses of the model are stable, and simulated AP waveforms closely resemble those observed in humans (Fig. 5c). We could now consider the effects of variable expression of TS channels in the model.

For G406R channels, which exhibit both enhanced activation gating and heightened opening in mode Ca, the effects were particularly severe. Upon initial increase of the fraction of G406R channels, the APD prolonged monotonically (Fig. 5d,e). However, upon further increase, we encountered a bifurcation of the APD in the model, where electrical responses became patently unstable (Fig. 5d,f). Interestingly, this region of beat to beat APD instability was achieved at levels just higher than those expected for TS1, and significant arrhythmogenesis would predominate at the higher levels of mutant channel expression anticipated for TS2 patients. Specifically, significant APD prolongation was seen at about 10% G406R expression, roughly corresponding to TS1 (heterozygous expression within exon 8a), while APD instability was seen by 20%.

By contrast, the effects of the G402S mutation, with its decreased propensity to open via activation gating and normal CDI\textsubscript{max}, appear to be substantially milder (Fig. 5g–i). Substantial APD prolongation does not arise until the fraction of mutant channels reaches the anticipated mean level of TS2 patients (~40% G402S versus WT as expected from a heterozygous...

**Figure 3** | Opposing shifts in channel activation in TS channels. (a) Exemplar on-cell current recordings in response to a voltage ramp (top). Channel openings are fit by the GHK equation (grey dashed line). Scale bar, 1 pA. (b) Addition of Bay K 8644 increases mode 2 openings, verifying channel number and GHK fit. (c) Current averaged over multiple patches gives the current voltage relation (black). The grey dashed relation is now the GHK relation scaled down by P_0/\text{max}. Scale bar, 0.2 pA. (d) P_0 as a function of voltage, averaged across cells (red) with Boltzmann fit (black). Grey displays ± s.e.m. G402S (middle) is clearly right shifted (case 1) compared with WT displayed as dashed line for comparison. G406R (right) shifts left (case 2) as compared with WT and G402S (dashed curves). n = 13, 8 and 7 for WT, G402S and G406R, respectively.
Figure 4 | Controlling the Ca\(^{2+}\) input reveals opposing mechanisms behind TS variants. (a) Ca\(^{2+}\) uncaging in Ca\(_{\text{v}}\)1.2 (E736A)\(^{27}\). Channels exhibit little inactivation with lithium as the charge carrier (black). Alexa568 and Fluo-4FF calcium dyes were dialyzed into cells, allowing simultaneous Ca\(^{2+}\) measurement (red). Internal Ca\(^{2+}\) was caged with DMNP-EDTA, allowing a step release of Ca\(^{2+}\) upon UV flash (cyan). Below, CDI is seen as a strong decrease in current (black) compared with pre-flash current (grey). Scale bar, 200 pA. (b) Lower levels of Ca\(^{2+}\) were achieved by varying the Ca\(^{2+}\) cage and UV flash intensity. (c) UV flash data obtained with CaM\(_{1234}\) yields the extent of residual Ca\(^{2+}\) pore block. (d) Fraction block produced by UV flash as a function of Ca\(^{2+}\) concentration for Ca\(_{\text{v}}\)1.2/CaM (black). Grey relation shows the residual pore block from CaM\(_{1234}\) experiments. Error bars indicate ± s.e.m. (e) The true CDI curve corrected for residual pore block. The curve for G402S (middle) is remarkably similar to WT Ca\(_{\text{v}}\)1.2 (left), resulting in a nearly identical CDI\(_{\text{max}}\) (red dashed line) while the CDI\(_{\text{max}}\) of G406R is significantly reduced (right panel, red dashed line).
mutation in exon 8). Interestingly, for TS2 patients, a slight increase in the fraction of G402S channels beyond the mean anticipated TS2 level is predicted to induce outright arrhythmia (at ~44%). Again, TS2 patients with the milder G402S mutation are nonetheless perched on the threshold of cardiac instability.

Direct test of model results in adult myocytes. The bifurcation phenomena identified by the LdR model hold important implications for pathogenesis and potential therapy. To test this prediction of the model more directly, we expressed WT, G406R, and G402S channels in cultured monolayers of adult guinea pig ventricular myocytes (aGPVMs), a preparation exhibiting APs with APDs comparable to human, making them suitable for exploring long QT phenomena. Recombinant channels were fused to GFP, allowing fluorescence measurements to report approximate expression levels. In this system, lentiviral-mediated expression of recombinant WT CaV1.2 channels caused only a slight prolongation of the cardiac APs compared with control cells (Fig. 6c). This lack of AP prolongation due to overexpression is consistent with previous reports that these myocytes may lower endogenous channel expression in response to exogenous expression, thus keeping the total number of channels relatively constant. This compensatory mechanism enables direct comparison of the aGPVM experiments with the model predictions.

Unlike WT channels, variable G406R expression (as quantified by GFP intensity), produced a marked effect on the APs. As in the LdR model, APs initially increased monotonically without appreciable variability (Fig. 6d,e). Upon slight further increase in G406R channels, however, APs became flagrantly unstable, as cells moved past a bifurcation point into a region of frank electrical instability (Fig. 6d,f). Hence, the instability threshold seen in the LdR model is confirmed in mammalian cardiac myocytes. Furthermore, as in our simulations, the G402S mutation produced a less pronounced APD prolonging effect. While low levels of expression did cause a slight increase in APD, significant APD prolongation by G402S (blue) did not arise, even past expression levels corresponding to a bifurcation point for G406R channels (Fig. 6g–i), confirming the ability of myocytes to withstand a higher load of G402S channels.

Figure 5 | Effects of opposing mechanisms on modelled action potentials. (a) The LdR model was used to gauge TS mutation effects on cardiac ventricular APs. Model activation curves (black curve) fit well the tail-current data (black circles). G406R (red) and G402S (blue) curves shifted to match experiments (circles). Inactivation parameters were similarly adjusted. (b) A single WT AP generated by the model. (c) Modelled WT APs displayed on an expanded timebase during 1-Hz pacing to show stability. (d) Severe effects of G406R channels. Variable G406R expression modelled by including a variable fraction of mutant versus WT channels. At first, APD increased monotonically, then became patently unstable before reaching TS2 levels. Asterisk indicates corresponding data in d. (f) Profound AP prolongation due to arrhythmogenic early after depolarizations at ~20% G406R. Double asterisk indicates corresponding data point in d. (g) Milder G402S mutation (blue) effects. Significant APD prolongation does not arise until mutant channels reach the anticipated mean of TS2 patients. The G406R curve is reproduced for comparison (dashed red). (h) APD prolongation at ~40% G402S. Asterisk indicates corresponding data in g. (i) Slight further increase of G402S (~44%) induces alternans. Double asterisk indicates corresponding data point in g.
Discussion

CDI represents a vital feedback mechanism required for proper Ca$^{2+}$ signalling in cardiac, neuronal and immune systems. Disruption of CDI in ventricular myocytes has been shown to cause substantial AP prolongation and similar defects in patients have now been discovered underlying a severe form of long QT syndrome. Yet the effects of the TS mutations on this vital form of channel regulation have remained unclear. Here, we have uncovered a significant effect of both TS mutations on the mechanisms underlying this loss of CDI. Such opposing mechanisms leading to the prolongation of the cardiac APs stand in contrast to the view that these mutations produce parallel effects in channel gating (primarily VDI), and thus lead to similar manifestations in patients. Rather, significant improvement may occur with only a small shift in the complement of mutant versus WT mutant channels. Rather, significant improvement may occur with only a small shift in the complement of mutant versus WT channels, either by specific pharmacological blockade or an alteration in channel expression.

The distinct effects of the TS mutations on channel gating give rise to a nonlinear dependence on TS gene expression. Importantly, the divergent mechanisms underlying the two TS mutations results in a significant difference in the amount of TS channel expression required to reach a threshold for the induction of arrhythmia. Given the implications of this nonlinear threshold behaviour, we replicated our in silico results in cultured monolayers of aGPVMs. The nonlinear threshold behaviour observed in the model is remarkably consistent with the behaviour observed in aGPVMs, confirming that the model predictions extend to networks of native myocytes. While our results predict that TS patients may reside on the cusp of an area of electrical instability such that patients are at high risk for severe arrhythmia. However, this study also offers new hope for patients. The nonlinear dependence of the APD on channel expression implies that it may not be necessary to block or correct all the mutant channels. Rather, significant improvement may occur with only a small shift in the complement of mutant versus WT channels, either by specific pharmacological blockade or an alteration in channel expression.

The in silico and live cell data presented in this study illustrate that the severity of TS is a function of both the specific mutation (G406R versus G402S) and the alternate exon in which it is expressed (exon 8 versus exon 8a). This dependence on gene
expression and mutation type correlates well with the severity of symptoms in TS patients. In the heart, the lower expression of the TS mutation in TS1 leads to a milder phenotype and increased patient survival. For G406R, this level of expression positions TS1 patients near an area of electrical instability. However, for patients harboring the G402S mutation, the lower expression level of TS1 may produce such a minimal phenotype that they may not exhibit overt symptoms, consistent with the lack of reported TS1 G402S patients. However, this potential population of patients should not be ignored, as it is possible that the more subtle effects on APD could put them at higher risk for acquired LQTS. For TS2, on the other hand, expression levels in patients are expected to be four times that of TS1 patients. For G406R, this level of expression would be expected to produce enormously increased QT durations and APD instability which would conspire to be lethal in most cases. This may rationalize the exceedingly small population of diagnosed G406R mutations in TS2 patients. It appears likely that strong compensatory mechanisms must arise in these TS2 patients during development to allow them to withstand the severe effects of the G406R mutation. For G402S, however, the higher TS2 gene expression levels again position patients near a threshold for electrical instability. The recent case reports have identified several patients with mosaic expression of a TS mutation. These patients are often identified among the healthy parents of TS children, indicating that the lower gene dosage induced by mosaic expression of the mutant gene may prevent outright cardiac symptoms, consistent with the predictions of this study.

In addition, these TS effects on APD may be accompanied by significant effects on EC coupling. In particular, the ventricular myocyte model predicts that both mutations cause a gene-dose-dependent increase in the amplitude of the Ca$^{2+}$ transient (Fig. 7). This effect is consistent with previous studies of the G406R mutation and is indicative of an increased SR Ca$^{2+}$ load in TS myocytes. Our results support the hypothesis that the TS channels produce an increase in the EC coupling gain. Importantly, this effect holds not only for the G406R channel, which exhibits a left shift in channel activation, but for the right-shifted G402S channel as well (Fig. 7), illustrating the critical role of Ca$^{2+}$ channel inactivation in EC coupling. Moreover, the altered Ca$^{2+}$ load due to TS channels may actually alter the function of the WT Ca$_V$1.2 channels within the cell. Specifically, a larger SR Ca$^{2+}$ release may increase the kinetics of CDI for WT Ca$_V$1.2 channels, while the lack of CDI in the mutant channels would tend to increase the steady state Ca$^{2+}$ current. This mixed population effect may explain why myocytes from TS mice have an overall increased rate of inactivation and a concurrent decrease in steady state inactivation. In all, gating defects of the TS channels appear to disrupt both the electrical properties and Ca$^{2+}$ handling within myocytes, leading to significant arrhythmogenesis.

Beyond these initial TS channels, the number of mutations identified in Ca$_V$1.2 is fast increasing. Many of these mutations produce symptoms which are quite similar to TS, while others lack a number of the TS features. This may be partially explained by the differing locations of these mutations. While many lie in similar S6 channel regions as the TS mutations, several have been identified in the N or C tails of the channel, as well as the II-III or I-II loop. Nonetheless, the effects of these mutations on CDI has been considered in only a few of these new cases. As the alteration of CDI may be a common feature of many Ca$^{2+}$ channelopathies, this may represent an important avenue of further investigation. As none of the recently reported Ca$_V$1.2 mutations are expressed in an exon known to undergo alternative splicing, it is possible that the particular biophysical defects of these channels may be tolerated at a higher level as compared with the TS mutations. Thus, studies such as this one, which span from single molecule biophysics to network level phenomenon, may enable a deeper understanding of the mechanisms underlying these channelopathies and provide insight into novel treatments for these patients.

More broadly, this nonlinear threshold phenomenon is likely not limited to TS. Indeed, there is reason to wonder whether channelopathies that most frequently attract clinical surveillance are those that pose patients on the threshold of outright instability. Channel mutations that position patients further from a domain of overt dysfunction may never garner clinical observation, while mutations that consign individuals to persistent and severe arrhythmias may curtail survival. It is therefore likely that the lessons learned from TS will shed new direction on a multitude of channelopathies.

**Methods**

**Molecular biology.** The human $\alpha_{1C}$ cardiac isoform was a kind gift from Tuck Wah Soong. A topo clone was generated spanning the front end of the channel from HindIII to Clal and the G406R and G402S mutations were introduced by quick change ligation (primer for G402R: CGTCTCAATCTGGTTCTCAGTG TGTGACCCAGAGTTTCCAAAG, primer for G402S: CGTCTCAATCTGGTTCTCAGTG TGTGACCCAGAGTTTCCAAAG). The construct was then transferred via HindIII and Clal into the full human $\alpha_{1C}$ backbone in pDNA3 (Acc# Z34810). For lentivirus expression, a GFP was attached to the C terminus of these channels at amino acid 1671 via introduction of an xba site at this location. The channels were then subcloned into the lentiviral plasmid pRRLsin18.CP.TMT.CMV.eGFP.Wpre which was a generous gift from Dr Gordon Tomasselli. Lentivirus production and purification were then carried out in Hek293T cells using a third generation lentiviral expression system.

**Figure 7 | TS effects on Ca$^{2+}$ transient amplitudes.** (a) The LRd model was used to gauge TS mutation effects on Ca$^{2+}$ transient amplitudes. Data corresponds to the same simulations run in Fig. 5. Increasing the fraction of G406R channels increased the average Ca$^{2+}$ transient amplitude (red circles). (+) indicates minimum and maximum amplitudes for an individual simulation. (b) Increasing the fraction of G402S channels also increased the average Ca$^{2+}$ transient amplitude (blue circles) but with a shallower dependence on the fraction of channels.
All flash experiments utilized a pore mutant which reduced Ca\(^{2+}\) pore block when patching in 80 mM Li\(^+\) external solution\(^{25,55}\). An E726A permeation mutant was introduced by overlap extension PCR on WT Ca\(_{V}1.2\). The E726A mutated fragment was then ligated into each channel construct using SrgAI and PpuMI. All sequences subject to PCR were verified by sequencing.

**Electrophysiology.** HEK293 cells (ATCC) were transfected using calcium phosphate\(^{26}\). Cells were co-transfected with 8 µg of rat brain β\(_{1b}\) or β\(_{2a}\) (ref. 57), 8 µg of rat brain β\(_{2a}\) (ref. 58), and 1–8 µg of Ca\(_{V}1.2\) channel 1α subunit and 2 µg of SV40 T-antigen; 8 µg of cDNA for rat brain CaM\(_{2}\) was added as required. A fluorophore was used in all transfections for identification of transfected cells. For standard whole-cell experiments, the beta subunit was contained in a GFP vector, while for Flash experiments, CFP was added to the transfection. CaM\(_{2}\) was contained within a CFPII vector so that cells with high levels of CaM\(_{2}\) could be distinguished. Room temperature, whole-cell recordings were performed 1–2 days after transfection, using Axopatch 200 A/B amplifiers (Axon Instruments).

P/8 leak subtraction was used, with series resistances of 1–2 MΩ after >70% compensation. Currents were filtered at 2 kHz (4-pole Bessel), and sampled at 10 kHz. For whole-cell experiments, internal solutions contained, (in mM): Ca\(_{2+}\) as above, MgCl\(_2\), 5; NaCl, 5; MgCl\(_2\), 5; EGTA, 2; glucose, 10; and HEPES (pH 7.4), 20 at 300 mOsm.

The concentration in the bath was

\[
\text{Ca}^{2+} = \text{Ca}_{2+}^{\text{max}} - \frac{R - \text{R}_{\text{meas}}}{\text{R}_{\text{max}} - R}
\]  

where \(R_2\) was determined to be 20 µM and \(R\) was the measured ratio of Fluor-4FF to Alexa568.

**Single-channel recordings.** On-cell recordings were obtained at room temperature (integrating mode, Axopatch 200B; Axon Instruments). Patch pipettes (3–10 MΩ) were pulled from ultra-thick-walled borosilicate glass (BF200-116-10, Sutter Instruments), and coated with Sylgard. Currents were filtered at 2 kHz. To zero membrane potential, the bath contained (in mM): K glutamate, 132; KCl, 5; NaCl, 5; MgCl\(_2\), 5; Na\(_2\)ATP, 5; HEPES, 10; and LiCl, 10. Data were analysed by custom MATLAB software (Mathworks, MA, USA), with average data shown as ± s.e.m. Ca\(_{2+}\) was calculated for each cell based on measured R\(_{\text{meas}}\) (40 mM EGTA) and R\(_{\text{max}}\) (1 mM EGTA).

Calcium solutions were made in the same base internal solution as above. Ca\(_{2+}\) concentration was calculated as:

\[
[C_{\text{a}}] = \text{K}_{2} \frac{R - \text{R}_{\text{meas}}}{\text{R}_{\text{meas}} - R}
\]  

where \(K_{2}\) was determined to be 20 µM and \(R\) was the measured ratio of Fluor-4FF to Alexa568.

**CDI measurements in aGPVMs.** To look for effects on CDI due to the G406R mutation within a native context, we utilized acute (<48 h post isolation) aGPVMs. As these cells are resistant to most transfection protocols, we adapted a Lipofectamine transfection method described for the transfection in adult rat ventricular myocytes\(^{4}\). Briefly, acutely dissociated myocytes were plated on laminin-coated coverslips for 1 h as described\(^{5}\). Once the cells had adhered to the substrate, the media was replaced with M199 media supplemented with 2 mM EGTA, 5 mM glucose, 2 mM L-Glutamine (Gibco), 5 mM HEPES, vitamin B12/penicillin (Sigma) and MEM non-essential amino acids (Gibco). After 4 h, cells were transfected with 6–12 µL Lipofectamine 2000 (Thermo Fisher) mixed in Opti-MEM (Gibco) with 3 µg Ca\(_{V}1.2\)-GFP (WT or G406R) and 2 µg T-antigen. The cells were incubated overnight at 37°C in 5% CO\(_2\) and rinsed with standard M199 media the next morning. Experiments were performed within 48 h of cell isolation. External solutions contained: (in mM): TEA-MeSO\(_3\), 140; HEPES (pH 7.4), 10; and LiCl, 80. Data were analysed by custom MATLAB software (Mathworks, MA, USA), with average data shown as ± s.e.m. Ca\(_{2+}\) was calculated for each cell based on measured R\(_{\text{meas}}\) (40 mM EGTA) and R\(_{\text{max}}\) (1 mM EGTA).

Formula for the voltage shift of each patch, and the activation curve (\(v\)). The unsubtracted traces, excluding blank sweeps, were averaged together, yielding an averaged trace. The key parameters, \(g\) and 2\(eV/RT\), were held constant for all patches of a given construct, only allowing \(V_{\text{dc}}\) to vary slightly from patch to patch. The leak-subtracted traces, excluding blank sweeps, were averaged together, yielding an \(I–V\) curve. For different patches, the leak subtracted traces were averaged together, compensated for the voltage shift of each patch, and the activation curve \(R_{\text{v}}\) versus \(V\) for each construct was determined using the HK equation\(^{29}\).

Following acquisition of sufficient traces, Bayk 8644 (Millicence, Pharmaceutical Div., West Haven, CT, USA) was added to the bath such that the final concentration in the bath was ~5 µM.

**Voltage protocol for flash.** Depolarizing voltage steps were preceded by a P/8 leak subtraction protocol and were recorded at 5 kHz Bessel filtering. Steps were applied to each cell at 30–60 s intervals and varied between 500 and 1,000 ms in duration to allow for the currents to reach steady state before and after initiation of the UV flash. UV flash was done between 50 and 300 ms following step depolarization, where the time was adjusted to allow for the slower activating G402S channel to reach a stable current amplitude before uncaging Ca\(_{2+}\). Because analysis was done relative to the time of UV flash these time adjustments did not alter our measurements, but instead insured that all data collected corresponded with steady state conditions. In addition, the surface charge change caused by use of Li\(^+\) in the external solution was accounted for by using a depolarizing step to 0 mV, which approximates (to the closest 10 mV increment) the 30 mV step done in 40 Ba\(^{2+}\) (Supplementary Fig. 8). To test this shift, experimental conditions for Ca\(_{2+}\) uncaging were approximately matched by using the same Li\(^+\) external solution, and 0.3 mM EDTA as an approximation of the DMNP-EDTA internal solution. The alteration in internal solutions was done simply as a practicality due to the cost of the DMNP-EDTA containing solution.

In addition to the adjustments in the amount of DMNP-EDTA Ca\(_{2+}\) cage, step size was varied by adjusting the UV flash lamp voltage within the range of 100–300 ms to change the capacitance of the ultraviolet source. Cells were cultured for a total of 3 weeks before optical mapping using 5 µM di-4-ANEPPS. During recording, cultures were paced at a frequency of 1 Hz and APs were recorded for 12 s from 253 sites using a custom built contact fluorescence imaging system\(^{39}\), which was generously made available to us by Dr Leslie Tung. Channels lacking a clear AP signal due to poor signal to noise were excluded. Ca\(_{2+}\) waveforms were averaged. AP\(_{\text{Dmax}}\) was measured from the average waveform, and s.d. was determined across the 12 s recording. To account for slight variations in different cultures, the AP\(_{\text{Dmax}}\) for TS expressing cells was normalized to control cells from the same culture.

**Quantification of Ca\(_{2+}\)1.2 lentiviral expression.** Lentiviral Ca\(_{V}1.2\) channels contained an attached GFP to allow quantification of relative channel expression. As aGPsVs some intrinsic autofluorescence, true GFP expression was determined using both GFP (excitation: 470 nm; emission: 530 nm) and CFP (excitation: 440 nm; emission: 480 nm) measurements as:

\[
\text{Relative expression} = \frac{\text{GFP} - \text{GFP}_{\text{autofluorescence}}}{\text{CFP} - \text{CFP}_{\text{autofluorescence}}}
\]

Where \(R = \text{GFP}_{\text{autofluorescence}}/\text{CFP}_{\text{autofluorescence}}\), was measured in uninfected aGPVMs each day. \(R_{2}\) was the ratio of GFP measured through the GFP cube compared with the CFP cube. This value was measured in HEK cells, which have minimal autofluorescence, transfected with GFP. \(R_{2}\) was determined to be 25 for our experimental setup. In all, this method allowed for both the removal of autofluorescence and normalization of monolayer thickness which was seen to vary between cultures.
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Author contributions
D.T.Y. and I.E.D. conceived the project, designed the experiments and created the figures. Molecular biology was done by W.Y. and I.E.D. I.E.D. performed the electrophysiological experiments and analysed the data. R.J.-M. isolated the myocytes and performed the optical recordings. D.T.Y. edited the LRd model, performed the simulations, and analysed the data. I.E.D. wrote the manuscript based on prior conversations with D.T.Y. and with comments from R.J.-M.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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