Cardiac-Specific Deletion of *Scn8a* Mitigates Dravet Syndrome-Associated Sudden Death in Adults

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Abstract

BACKGROUND—Sudden unexpected death in epilepsy (SUDEP) is a fatal complication experienced by otherwise healthy epilepsy patients. Dravet syndrome (DS) is an inherited epileptic disorder resulting from loss of function of the voltage-gated sodium channel, Na_V 1.1, and is associated with particularly high SUDEP risk. Evidence is mounting that Na_Vs abundant in the brain also occur in the heart, suggesting that the very molecular mechanisms underlying epilepsy could also precipitate cardiac arrhythmias and sudden death. Despite marked reduction of Na_V 1.1

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APPENDIX For an expanded Methods section as well as a supplemental table and figures, please see the online version of this paper.

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

functional expression in DS, pathogenic late sodium current ($I_{Na,L}$) is paradoxically increased in DS hearts. However, the mechanisms by which DS directly impacts the heart to promote sudden death remain unclear.

OBJECTIVES—In this study, the authors sought to provide evidence implicating remodeling of Na^+-and Ca^{2+}-handling machinery, including $Na_v 1.6$ and Na^+/Ca^{2+} exchanger (NCX) within transverse (T)-tubules in DS-associated arrhythmias.

METHODS—The authors undertook scanning ion conductance microscopy (SICM)-guided patch clamp, super-resolution microscopy, confocal Ca^{2+} imaging, and in vivo electrocardiography studies in *Scn1a* haploinsufficient murine model of DS.

RESULTS—DS promotes $I_{Na,L}$ in T-tubular nanodomains, but not in other subcellular regions. Consistent with increased Na_V activity in these regions, super-resolution microscopy revealed increased $Na_V 1.6$ density near Ca^{2+} release channels, the ryanodine receptors (RyR2) and NCX in DS relative to WT hearts. The resulting $I_{Na,L}$ in these regions promoted aberrant Ca^{2+} release, leading to ventricular arrhythmias in vivo. Cardiac-specific deletion of $Na_V 1.6$ protects adult DS mice from increased T-tubular late Na_V activity and the resulting arrhythmias, as well as sudden death.

CONCLUSIONS—These data demonstrate that $Na_V 1.6$ undergoes remodeling within T-tubules of adult DS hearts serving as a substrate for Ca^{2+}-mediated cardiac arrhythmias and may be a druggable target for the prevention of SUDEP in adult DS subjects.

Keywords

Dravet syndrome; $Na_V 1.6$; sodium channels; sudden cardiac death; sudden unexpected death in epilepsy

Sudden unexpected death in epilepsy (SUDEP) is a fatal complication, occurring in patients with epilepsy who were otherwise healthy. Patients with inherited epileptic disorders, such as Dravet syndrome (DS), are at a particularly high risk of sudden death.[1] At the subcellular level, DS is characterized by the loss of function or expression of a specific voltage-gated sodium channel (Na_V), $Na_V 1.1$, encoded by the *SCN1A* gene.[2] In SUDEP cases where an autopsy is performed, no clear cause of death is found.[3] SUDEP shares this feature with cases of sudden cardiac death—unexpected death resulting from cardiac arrhythmias. Prior work on the cardiovascular contribution to SUDEP has largely focused on autonomic modulation of cardiac function.[4,5] However, therapies based on this notion have demonstrated limited efficacy in preventing SUDEP.[6] Na_Vs expressed at high levels in the brain (commonly labeled "neuronal-type" Na_Vs) are also expressed in cardiomyocytes.[7–10] Further, DS-causing mutations in *SCN1A* have been shown to alter cardiomyocyte function in both preclinical animal models and induced pluripotent stem cells derived from patients with DS.[11,12] Taken together, these studies suggest that the molecular mechanisms underlying the epileptic condition in the brain may also directly affect the heart, independent of autonomic control, predisposing the epileptic heart to arrhythmias and sudden cardiac death.

Although DS is characterized by a marked reduction of functional $Na_V1.1$, previous studies have demonstrated a paradoxical increase in the late component of the voltage-gated sodium current in these hearts ($I_{Na,L}$).[11] At present, however, the precise mechanisms by which $Na_V1.1$ loss of function leads to these paradoxical changes in $I_{Na,L}$ and how these, in turn, contribute to arrhythmia remain unknown.[11,13] Further, although many investigations of SUDEP in DS have focused on death in the pediatric population, which may reflect the availability of preclinical models with a specific focus on early mortality rather than the clinical scenario in many DS patients. There is a precipitous loss of life among DS patients not only during early childhood but also during late adolescence and early adulthood.[14–16] The second wave of mortality associated with DS in early adulthood remains understudied.

In this study, we directly address this gap in knowledge using an adult (>100 days of age) murine model of DS to study how *Scn1a* haploinsufficiency contributes to cardiac arrhythmias. Mechanistically, we demonstrate that DS results in an increase in functional $Na_V1.6$ expression within transverse tubule (T-tubule) nanodomains of cardiomyocytes, which promotes ectopic Ca^{2+} release at the cellular level and arrhythmias in vivo. We further demonstrate that a cardiac-specific reduction in *Scn8a* (encoding $Na_V1.6$) is sufficient to reduce arrhythmia burden and improve survival in DS mice. Collectively, these data strongly suggest that maladaptive remodeling of $Na_V1.6$ within the heart results in sudden death, which contributes to mortality in adult DS. These results highlight cardiac $Na_V1.6$ as a potential therapeutic target for preventing sudden death in DS.

METHODS

A detailed description of the methods is provided in the Supplemental Appendix.

STATISTICS.

Experiments and analyses were conducted in an unblinded fashion. We performed all statistical analyses using GraphPad Prism 9.4.1. To test for data normality, we performed the Shapiro-Wilk test. To compare 2 independent data sets, we performed the unpaired 2-tailed Student's *t*-test or the Mann-Whitney *U* test where appropriate, depending on the normality of the data. Differences in distributions were tested with the 2-sample Kolmogorov-Smirnov test. A *P* value of <0.05 was considered significant. To compare more than 2 data sets, we performed an ordinary 1-way analysis of variance or Kruskal-Wallis test depending on the normality of the data. To correct for multiple comparisons, we performed a post hoc analysis with the original method of Benjamini and Hochberg. A *q* value of <0.05 was considered significant. All data in this report are presented as the mean ± SEM or as box-and-whisker plots. For survival analysis, curves were compared using the log-rank (Mantel-Cox) test and corrected for multiple comparisons post hoc via Tukey's method.

STUDY APPROVAL.

All animal procedures were approved by Institutional Animal Care and Use Committee at The Ohio State University and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication no. 85–23, revised 2011).

RESULTS

CARDIAC-SPECIFIC ABLATION OF *SCN8A* REDUCES ALL-CAUSE MORTALITY AND ARRHYTHMIAS IN DS.

Consistent with previous findings in mice with DS, we observed a ~20% mortality in our cohort within the first 60 days.[17] Notably, a second wave of sudden death was apparent after 100 days (Figure 1A), consistent with mortality trends observed in patients with DS when adjusted for species-dependent age differences.[14–16,18] Importantly, this mortality phenotype was independent of the mouse strain used in this study (Supplemental Figure 1). Previous studies have demonstrated that reducing $Na_V1.6$ (encoded by *Scn8a*) can ameliorate seizure burden and prevent sudden death in DS.[19–22] In line with these findings, we observed that *Scn8a* haploinsufficient DS mice (DSxNa$_V$1.6^{Het}) are protected from sudden death (Figure 1A, Supplemental Figure 1). To test for a role of cardiac $Na_V1.6$ in DS arrhythmia development, we crossed DS mice with a previously validated cardiac-specific $Na_V1.6$-knockout mouse (DSxcNa$_v$1.6^{KO}).[23,24] This strategy reduced mortality relative to DS (Figure 1A, Supplemental Figure 1). In contrast to global reduction in $Na_V1.6$ expression (DSxNa$_V$1.6^{Het}), cardiac-specific $Na_V1.6$ deletion (DSxcNa$_V$1.6^{KO}) did not confer protection from sudden death within the first 60 days but substantially mitigated sudden death in the adult population. Our findings thus suggest mechanistic differences in mortality in early childhood vs adulthood in DS, implicating a primary cardiac defect in excitability and consequent sudden death as a driver of mortality in the latter subpopulation. Consistent with previous findings,[11] adult DS mice displayed QT-interval prolongation relative to the wild type (WT), an established marker of arrhythmia susceptibility (Figures 1B and 1C).[25] Conversely, adult DSxNa$_V$1.6^{Het} and DSxcNa$_V$1.6^{KO} evidenced QT intervals comparable to those observed in WT mice, suggesting that changes in functional expression of cardiac $Na_V1.6$ contribute to the long-QT-interval phenotype observed in DS (Figure 1C). Our observation of QT-interval prolongation, coupled with reports of SUDEP during exercise[14,15] and sympathetic dominance in the DS population[26] prompted us to further investigate the arrhythmia susceptibility of adult DS mice with an in vivo catecholamine challenge. Our studies show that adult DS mice have a significantly higher catecholamine-induced arrhythmia burden than WT mice. Importantly—and consistent with the hypothesis that changes in functional expression of cardiac $Na_V1.6$ in DS contribute to cardiac arrhythmias—arrhythmia burden during catecholamine challenge was significantly reduced in both adult DSxNa$_V$1.6^{Het} and DSxcNa$_V$1.6^{KO} mice (Figures 1D and 1E). Taken together, our in vivo findings suggest that $Na_V1.6$ is a critical component of arrhythmogenesis that contributes to sudden death in DS mice.

Na$_V$1.6 CONTRIBUTES TO LATE Na$_V$ ACTIVITY WITHIN CARDIAC T-TUBULAR NANODOMAINS.

Recent reports have linked DS with a paradoxical increase in $I_{Na,L}$,[11,12] which may contribute to the increased arrhythmia burden, as observed in our DS mice (Figures 1D and 1E). To further investigate this, we measured $I_{Na,L}$ in DS hearts. Consistent with previous reports, we observed a significant increase in whole-cell $I_{Na,L}$ in cardiomyocytes isolated from DS mice (Figure 2A, Supplemental Figure 2A). In contrast, no changes were observed

in peak I_{Na} (Figure 2B), I_{Na} activation, or I_{Na} inactivation kinetics (Supplemental Figures 2B and 2C).

The heart expresses various Na_V isoforms with distinct patterns of subcellular distribution. The predominant cardiac Na_V isoform, $Na_V1.5$, is heavily enriched at cardiac cell-cell junctions, known as intercalate discs (IDs), with some also localized to the lateral membrane and T-tubules (Figure 3A, Supplemental Figure 3).[24,27,28] Meanwhile, $Na_V1.1$ and $Na_V1.6$ are predominantly located near Ca^{2+}-handling proteins within T-tubules (Figure 3A).[8,24,29,30] To gain insight into the molecular identity and distribution of the Na_V responsible for the increased $I_{Na,L}$, we first directly assessed Na_V activity using cell-attached patch clamp at the ID (Figure 3B). There were no significant differences in Na_V activity at the ID between WT and DS cardiomyocytes (Figure 3C). One key limitation of the conventional cell-attached patch clamp approach is the inability to place the pipette at specific subcellular regions and thereby discriminate between subcellular features. Therefore, to examine Na_V activity at the lateral membrane crest as well as at the T-tubules, we used the scanning ion-conductance microscopy (SICM)–guided "smart" patch clamp approach.[24] Similar to the ID, Na_V activity at the crests of the lateral membrane (Figure 3D) did not reveal significant differences in late Na_V activity between DS and WT (Figure 3E). Conversely, smart patch recordings from T-tubular regions (Figure 4) revealed increased late Na_V activity in DS cardiomyocytes relative to WT cardiomyocytes (Figure 4A), which was significantly reduced in both DSxNa$_V1.6^{Het}$ and DSxcNa$_V1.6^{KO}$ (Figure 4B). Together, these subcellular findings suggest that $Na_V1.6$ within T-tubules is the main source of excess $I_{Na,L}$ in DS cardiomyocytes.

$Na_V1.6$ IS REMODELED WITHIN T-TUBULAR CA^{2+}-HANDLING NANODOMAINS IN DS.

Our findings from subcellular electrophysiology assays suggest that the paradoxical increase in $I_{Na,L}$ in DS is a result of increased $Na_V1.6$ activity within the T-tubules. Consistently, Western blots of DS hearts revealed an increase in total $Na_V1.6$ protein relative to WT (Figures 5A and 5B, Supplemental Figure 4A) without concomitant alterations to the expression or localization of the predominant cardiac Na_V isoform, $Na_V1.5$ (Supplemental Figures 4B and 4C and 3, respectively). Although supportive of our hypothesis, Western blotting provides no information on the subcellular region that evidences change in $Na_V1.6$ expression. Based on our SICM-guided smart patch experiments, we expect Ca^{2+}-handling machinery-rich regions of T-tubules to harbor these changes. Therefore, to confirm the subcellular localization of changes in $Na_V1.6$ expression, we performed proximity ligation assays. Our data demonstrate an increase in $Na_V1.6$-ryanodine receptors (RyR2) and $Na_V1.6$-sodium/calcium exchanger (NCX) puncta in DS mice relative to WT (Supplemental Figures 5A and 5B). To further assess $Na_V1.6$ nanodomain spatial organization, we performed superresolution imaging, specifically stochastic optical reconstruction microscopy, which enables assessment of protein distribution in cardiomyocytes with ~20-nm lateral and <50-nm axial resolution.[31] Analysis with a previously validated machine learning-based cluster analysis tool (STORM-RLA[31]) revealed that in DS hearts, $Na_V1.6$ is in closer proximity to both RyR2 and NCX than in WT (Figure 6). These results suggest that Ca^{2+}-handling nanodomains within

T-tubules of DS hearts are further enriched with $Na_V1.6$, which may provide a structural substrate for the aberrant Ca^{2+} release that underlies in vivo arrhythmias and sudden death.

Next, we performed Monte Carlo simulations to gain insight into the potential mechanism responsible for changes in $Na_V1.6$ distribution vis-à-vis Ca^{2+}-handling machinery within the T-tubules. Using a model that assumes channel-channel interactions via passive biophysics without recourse to active biochemical processes (see Methods), we were able to replicate the pattern of clustering between NCX and $Na_V1.6$ (Figure 7) that we observed experimentally in WT hearts (Figure 6). Importantly, simulations of DS (partial $Na_V1.1$ reduction) result in an increase of $Na_V1.6$ near NCX (Figures 7B to 7D), and the distribution of NCX near $Na_V1.6$ was unchanged, paralleling the experimental findings (Figure 6, Supplemental Figure 6). The computational results, therefore, led us to hypothesize that channel-channel functional interactions may be sufficient to explain the compensatory changes in $Na_V1.6$ cluster distribution in DS without recourse to other compensatory mechanisms. To test this further, we assessed *Scn8a* transcript levels in WT and DS hearts. Consistent with the hypothesis derived from numerical simulations, we did not detect significant changes in *Scn8a* messenger RNA levels between WT and DS hearts (Supplemental Figure 4D). Furthermore, in line with previous observations,[32] these results together suggest the hypothesis that channel-channel functional interaction between $Na_V1.6$ and $Na_V1.1$ may help shape the cluster size of $Na_V1.6$, which in turn can effect nanodomain ionic composition.

$Na_V1.6$ REMODELING IN DS PROMOTES PROARRHYTHMIC CA^{2+} RELEASE.

To examine the functional consequences of $Na_V1.6$ remodeling within Ca^{2+}-handling nanodomains on Na^+/Ca^{2+} exchange and proarrhythmic Ca^{2+} release, we performed confocal line scan Ca^{2+} imaging. Confocal Ca^{2+} imaging revealed a significant increase in the frequency, amplitude, and duration of Ca^{2+} sparks in DS cardiomyocytes relative to WT (Figures 8A and 8B, Supplemental Figure 7D), without affecting other Ca^{2+} spark parameters or sarcoplasmic reticulum Ca^{2+} load (Supplemental Figure 7). Likewise, we observed a significant increase in spontaneous Ca^{2+} wave frequency (Figure 8C). Consistent with the involvement of $Na_V1.6$ in proarrhythmic Ca^{2+} release, Ca^{2+} spark and Ca^{2+} wave frequency were reduced in $DSxNa_V1.6^{Het}$ and $DSxcNa_V1.6^{KO}$ hearts relative to DS. Together, our data suggest that structural remodeling of $Na_V1.6$ within Ca^{2+}-handling-rich nanodomains underlies the development of cardiac arrhythmias that contributes to sudden death in adult DS mice, presenting $Na_V1.6$ as a viable therapeutic target.

DISCUSSION

Patients with inherited epileptic disorders, such as DS, are at a particularly high risk of sudden death.[1] Several population-level studies investigating SUDEP in epileptic populations have indicated cardiorespiratory arrest as one of the leading causes of death.[33,34] This has been supported by findings in murine models of DS.[5,35,36] It is important to note that these studies have focused on postictal SUDEP in the young DS subpopulation. Similarly, previous studies investigating the cardiac component of SUDEP have focused on how an epileptic brain may dysregulate the heart via autonomic input during the

peri-ictal period.[4,5,11] To our knowledge, studies investigating the causes for non-seizure-related sudden death in adult DS models are lacking. Importantly, however, many epilepsy-causing mutations occur in proteins that are endogenously expressed not only in the brain but also in the heart. This includes several voltage-gated ion channels integral to ionic homeostasis, including multiple Na_v isoforms.[8,9,24,29,30] Based on this rationale, we postulated that sudden death associated with DS is, in part, a consequence of direct dysregulation of cardiac physiology by mutated proteins expressed in the heart. Recently, we and others have demonstrated that alterations in tetrodotoxin-sensitive Na_Vs ($Na_V1.1$ and $Na_V1.6$) directly promote cardiac arrhythmias in vivo by disrupting Na^+/Ca^{2+} cycling at the subcellular level.[7,24,29,37–40] Here, we show, for the first time to our knowledge, that *Scn1a* haploinsufficiency results in structural remodeling of $Na_V1.6$ within cardiac T-tubules, a microdomain rich in Na^+/Ca^{2+}-handling proteins. Maladaptive remodeling of $Na_V1.6$ promotes excessive Na_V activity in close proximity to NCX, resulting in ectopic Ca^{2+} influx, which then triggers arrhythmogenic Ca^{2+} release from intracellular stores. Ultimately, $Na_V1.6$ remodeling predisposes the animal to arrhythmia development and sudden death. We further demonstrate that cardiac-specific deletion of $Na_V1.6$ is sufficient to significantly mitigate both arrhythmia and mortality in adult DS mice.

SUDDEN CARDIAC DEATH IN DS.

Clinically, sudden death in patients with DS first manifests in early childhood (<4 years old), followed by a second peak in mortality during the transition from late adolescence into early adulthood (~18 years old).[14–16] With adjustment for age differences between the 2 species,[18] the DS murine models used in this study phenocopy both the pediatric and early-adult sudden death trends in clinical populations (Figure 1A, Supplemental Figure 1). Historically, the abnormally high incidence of sudden death in DS pediatric populations has been attributed to postictal generalized electroencepha-logram suppression,[41] respiratory arrest,[35,42] acute cardiac bradycardia followed by asystole,[5,11] or hyperthermia-induced ventricular tachycardia.[13] Because sudden death in adult DS models has been understudied, little is known about the potential etiologies of sudden death in adult DS populations.[14–16]

Despite not having a clear understanding of how death occurs in adult DS, multiple studies have demonstrated that suppressing $Na_V1.6$ can reduce mortality in murine models of DS.[19–22] However, it remains unclear to what extent $Na_V1.6$ inhibition can rescue cardiac dysfunction in DS models. To answer this question, we genetically ablated $Na_V1.6$ from the hearts of our DS mouse model (DSxc$Na_V1.6^{KO}$). Early in life, the mortality in our DSxc$Na_V1.6^{KO}$ model parallels DS mice. Unlike the DS mice, however, our DSxc$Na_V1.6^{KO}$ mice do not experience a second wave of death in adulthood. Our data thus support the notion that DS-related deaths during adulthood may be, in part, a direct consequence of endogenous cardiac dysfunction, whereas those in infancy and childhood are likely caused by defects in the central or autonomic nervous system and their effect on downstream organ systems.[35,42]

In support of the hypothesis that cardiac death contributes to sudden death in DS, it is well established that DS results in altered cardiac physiology.[5,11–13] At the level of an isolated cardiomyocyte, there is an increase in cardiomyocyte excitability and $I_{Na,L}$, both of which

would predispose the heart to the development of arrhythmias.[11] In the DS population, QT-interval prolongation has been documented, though it must be noted that this cohort did not evidence major ventricular arrhythmias during the peri-ictal period.[43] Additionally, DS mice in our study were not observed via video monitoring; therefore, the precise mechanism of death remains unknown. It is therefore difficult to ascertain whether death in DS mice was preceded by seizure, post-ictal apnea and bradycardia, cardiac tachyarrhythmia, or another sentinel event. However, the significant increase in survival that accompanies cardiac-specific $Na_V1.6$ ablation suggests that the heart contributes significantly to mortality in our adult DS models (Figure 1).

One note of particular interest is that population-level studies of DS-associated SUDEP reveal accidental death by drowning as a recurrent etiology.[14,15] These reports hint that exercise and physical exertion coupled with increased sympathetic tone may precipitate sudden death in these patients. Consistent with this hypothesis, prior studies have demonstrated sympathetic dominance in adult DS patients, secondary to a reduction in parasympathetic tone.[26,43,44] Further, sympathetic dominance has long been linked to increased susceptibility to lethal ventricular arrhythmias that underlie sudden cardiac death.[45] To test whether adult DS mice are more susceptible to sympathetically induced arrhythmias, we subjected anesthetized mice to an in vivo catecholamine challenge and observed a significant increase in arrhythmia burden associated with DS. Further, consistent with our overall mortality data, we found that a cardiac-specific deletion of $Na_V1.6$ significantly decreased the arrhythmia burden in these mice.

Other causes of death, such as respiratory dysfunction before bradycardia, have been proposed as possible contributors to SUDEP.[35,42] These studies suggest that brainstem respiratory centers could be affected to compromise respiration under interictal conditions. However, evidence from mice with *Scn1a* haploinsufficiency in forebrain gamma-aminobutyric acid interneurons points to dysfunction in the autonomic nuclei in the brainstem that regulate cardiac and other essential functions as drivers of primary bradycardia promoting SUDEP in DS mice.[5] These findings collectively suggest that multifactorial etiologies may contribute to SUDEP in DS. Importantly, our study adds remodeling of $Na_V1.6$ channels in the heart as an additional key contributor to sudden death in DS.

DYSREGULATION OF CARDIAC $Na_V1.6$ UNDERLIES PROARRHYTHMIC CA^{2+} RELEASE.

A majority of previous work investigating the cardiovascular contribution to SUDEP has focused on altered autonomic modulation of the heart. However, emerging evidence suggests that the very molecular mechanisms that underlie epileptic disorders could also directly affect the heart, leading to arrhythmias and sudden death.[46] Until now, however, the mechanisms by which DS may promote sudden cardiac death remained unclear. To gain mechanistic insight into DS-associated cardiac dysfunction, we used an SICM-guided smart patch to study Na_V activity in different nanodomains of the cardiomyocyte. Doing so, we determined that there was an increase in Na_V activity within the T-tubules but not in other subcellular compartments, such as the lateral membrane crest or the intercalated disc.

Prior work by us and others has demonstrated that Na_V clusters within the T-tubules are composed mainly of $Na_V1.1$ and $Na_V1.6$.[8–10,23,37] Given that DS is associated with a functional reduction in $Na_V1.1$, our data suggest that increased T-tubular $Na_V1.6$, although apparently small but in such a location, exerts a potent functional effect, which gives rise to the paradoxically increased late Na_V activity within these nanodomains. Excess late Na_V activity, in turn, engages NCX and results in spontaneous Ca^{2+} waves, which underlie cardiac arrhythmias.

Notably, using superresolution microscopy and electrophysiology, coupled with computational modeling and protein and transcript analysis, we demonstrate that $Na_V1.6$ increases in proximity to Ca^{2+}-handling machinery (RyR2 and NCX), compensating for the loss of $Na_V1.1$ in DS (Figure 9). This notion is consistent with previous experimental observations that have demonstrated an inhibitory effect of $Na_V1.1$ on $Na_V1.6$ functional expression.[32] It is important to note that changes in T-tubular $Na_V1.6$ could be a result of enhanced messenger RNA trafficking and local translation, reduced $Na_V1.6$ degradation, or both and require further investigation. Importantly, the relevance of $Na_V1.6$ in the arrhythmogenic process was further confirmed through germline $Na_V1.6$ reduction and cardiac-specific $Na_V1.6$ knockout, both of which prevented ectopic Ca^{2+} release and lowered the resulting arrhythmias and sudden death in DS.

CONCLUSIONS

We provide here the first direct evidence, to our knowledge, that dysregulation of the heart significantly contributes to death in this adult murine model of DS. We demonstrate that *Scn1a* haploinsufficiency results in remodeling of $Na_V1.6$ in cardiac T-tubules, which drives aberrant Ca^{2+} release, promotes arrhythmogenesis, and ultimately results in untimely death (Central Illustration). We further demonstrate that cardiac-specific ablation of *Scn8a* is sufficient to rescue the cardiac arrhythmia phenotype and consequent mortality observed in adult DS mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

FUNDING SUPPORT AND AUTHOR DISCLOSURES

ABBREVIATIONS AND ACRONYMS

cNa$_V$ 1.6^{KO}	cardiac-specific Na$_V$1.6 deletion
DS	Dravet syndrome
ID	intercalate disc

$I_{Na,L}$	voltage-gated sodium current
Na_V	voltage-gated sodium channel
$Na_V1.6^{Het}$	*Scn8a* haploinsufficient
NCX	sodium/calcium exchanger
RyR2	ryanodine receptors
SICM	scanning ion-conductance microscopy
SUDEP	sudden unexpected death in epilepsy
T-tubule	transverse tubule
WT	wild type

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE:

Our results highlight a previously unappreciated role for the remodeling of sodium channels, including $Na_V1.6$, in cardiac T-tubular nanodomains and their contribution to sudden death in DS.

TRANSLATIONAL OUTLOOK:

Given that $Na_V1.6$ undergoes remodeling within T-tubules of DS hearts, serving as a substrate for Ca^{2+}-mediated cardiac arrhythmias, $Na_V1.6$ may be a druggable target for the prevention of sudden death in DS.
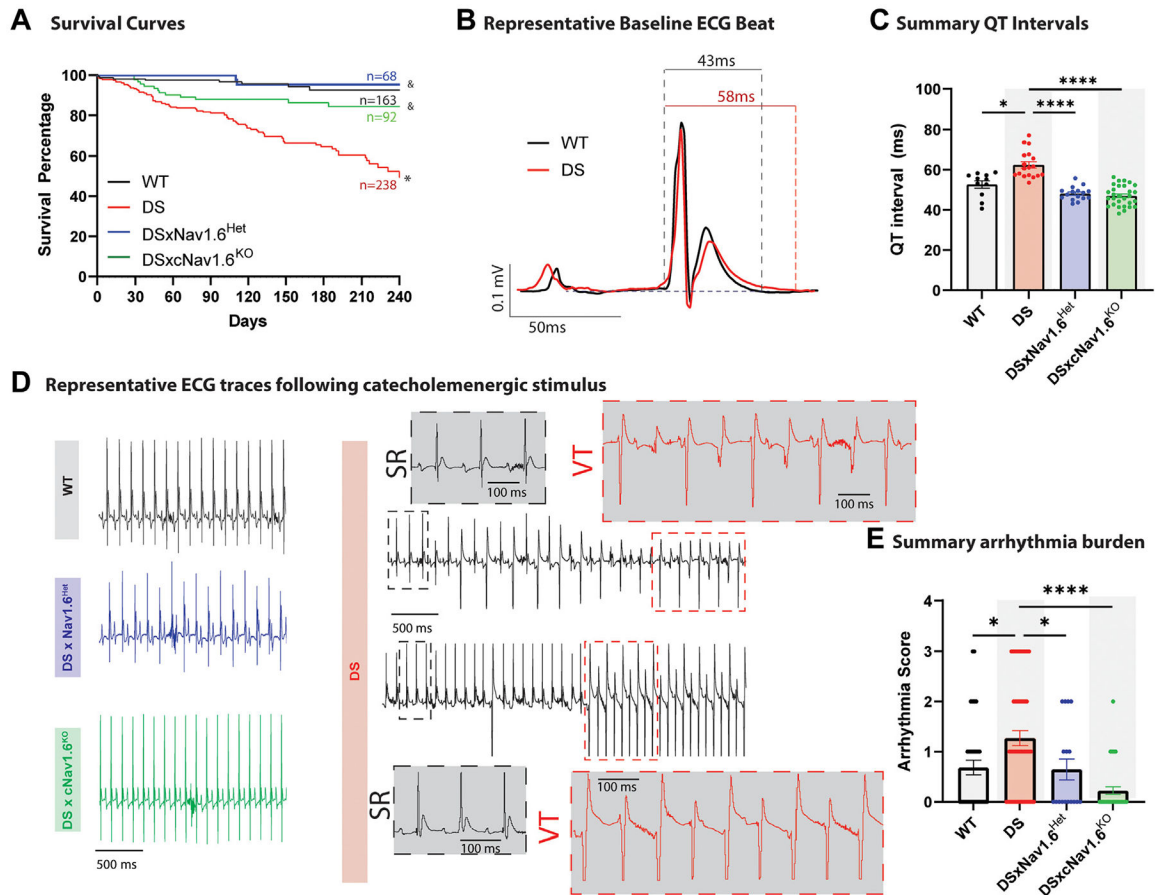
FIGURE 1. DS Is Associated With an Increase in Mortality and Predisposition to Arrhythmia Development, Which Are Mitigated by Reduction in Na$_V$1.6

(A) Kaplan-Meier survival curves for WT (n = 163), DS (n = 238), DSxNa$_V$1.6^{Het} (n = 68), and DSxcNa$_V$1.6^{KO} (n = 92) mice. The data from all strains tested for WT and DS were pooled. *P< 0.001 relative to WT, $^\&P$< 0.001 relative to DS. (B) Representative ECG from WT and DS mice. (C) QT-interval prolongation in DS, mitigated by Na$_V$1.6 reduction (WT: n = 11; DS: n = 18; DSxNa$_V$1.6^{Het}: n = 17; DSxcNa$_V$1.6^{KO}: n = 29 mice). (D) Representative ECGs following catecholamine challenge with caffeine and epinephrine illustrate SR followed by VT. (E) Summary data showing significant increase in arrhythmia burden in DS mice (WT: n = 38; DS: n = 59; DSxNa$_V$1.6^{Het}: n = 17; DSxcNa$_V$1.6^{KO}: n = 40). Arrhythmia scores: 0 = no abnormalities; 1 = premature ventricular complexes observed; 2 = bigeminy; 3 = VT; 4 = ventricular fibrillation. *q< 0.05, ****q< 0.0001 relative to DS. DS = Dravet syndrome; ECG = electrocardiogram; Na$_V$ = voltage-gated sodium channel; cNa$_V$1.6^{KO} = cardiac-specific Na$_V$1.6 deletion; Na$_V$1.6^{Het} = *Scn8a* haploinsufficient; SR = sinus rhythm; VT = ventricular tachycardia; WT = wild type.
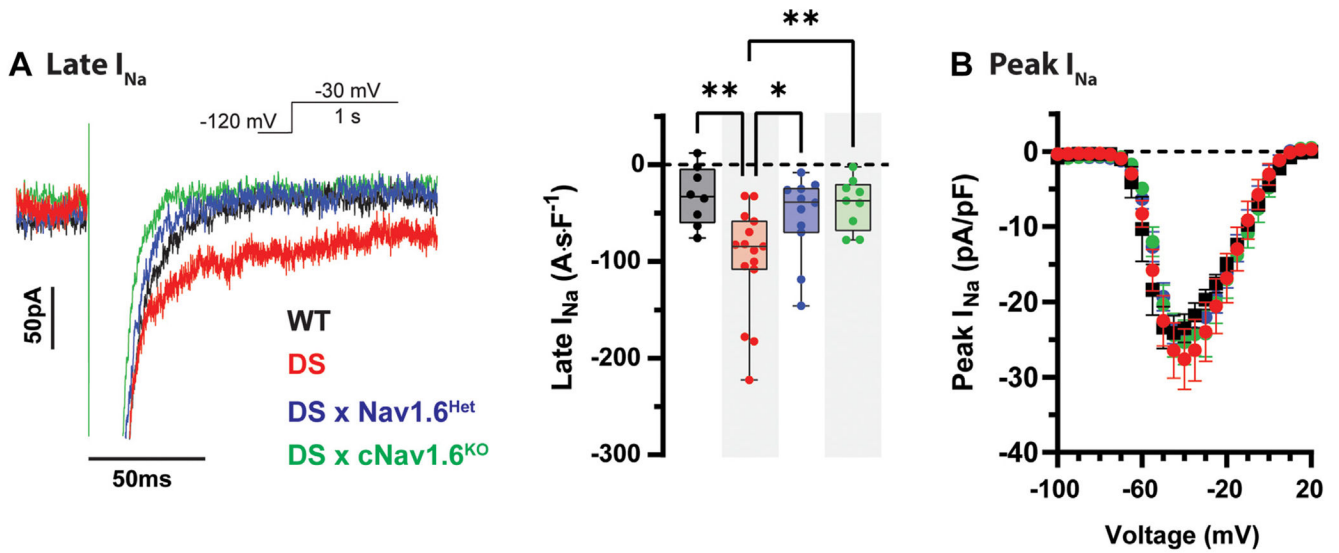
FIGURE 2. Whole-Cell Patch Clamp Recordings Reveal Increased Late I_{Na} in DS Cardiomyocytes, Which Are Mitigated by Reduction in $Na_V1.6$

(A) Representative late I_{Na} recordings from cardiomyocytes of each experimental group; increased late I_{Na} in DS cardiomyocytes is mitigated by $Na_V1.6$ reduction (WT: n = 10 cells, 4 mice; DS: n = 15 cells, 8 mice; DSxNa$_V$1.6^{Het}: n = 13 cells, 6 mice; DSxcNa$_V$1.6^{KO}: n = 9 cells, 5 mice). (B) Peak I_{Na} (WT: n = 5 cells, 3 mice; DS: n = 8 cells, 7 mice; DSxNa$_V$1.6^{Het}: n = 9 cells, 5 mice; DSxcNa$_V$1.6^{KO}: n = 12 cells, 4 mice). *$q < 0.05$, **$q < 0.01$ relative to DS. $I_{Na,L}$ = voltage-gated sodium current; other abbreviations as in Figure 1.
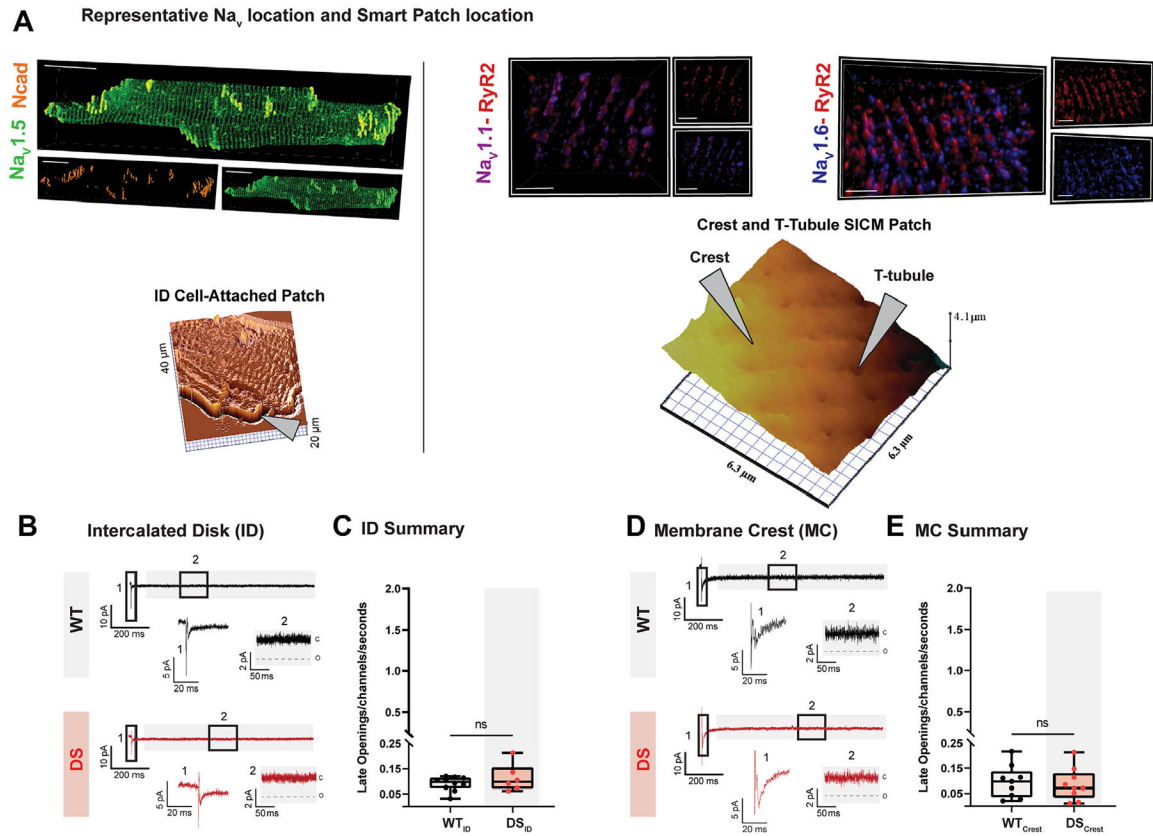
FIGURE 3. No Changes in Late Na_V Activity Within the Intercalated Disc or the Lateral Membrane Crests of DS Cardiomyocytes

(A, top) Representative immunofluorescence confocal microscopy images of Na_V ($Na_V1.5$: green; $Na_V1.1$: purple; $Na_V1.6$: blue) distributions relative to ID (N-cadherin: orange) and T-tubular (RyR2: red) regions and (bottom) representative scanning ion conductance microscopy images of cardiomyocyte topography (specific regions indicated by markers; left ID, right crest and T-tubule). Scale bars: (left) 20 μm and (right) 2 μm. (B) Cell-attached patch I_{Na} recordings from the ID. (D) Scanning ion conductance microscopy-guided "smart" patch I_{Na} recordings from the lateral membrane crest. (C and E) Summary data of late activity at the ID (WT: n = 10 cells, 3 mice; DS: n = 6 cells, 3 mice) and later membrane crest (WT: n = 9 cells, 3 mice; DS: n = 9 cells, 3 mice). ID = intercalate disc; ns = not significant; RyR2 = ryanodine receptors; T-tubule = transverse tubule; other abbreviations as in Figures 1 and 2.
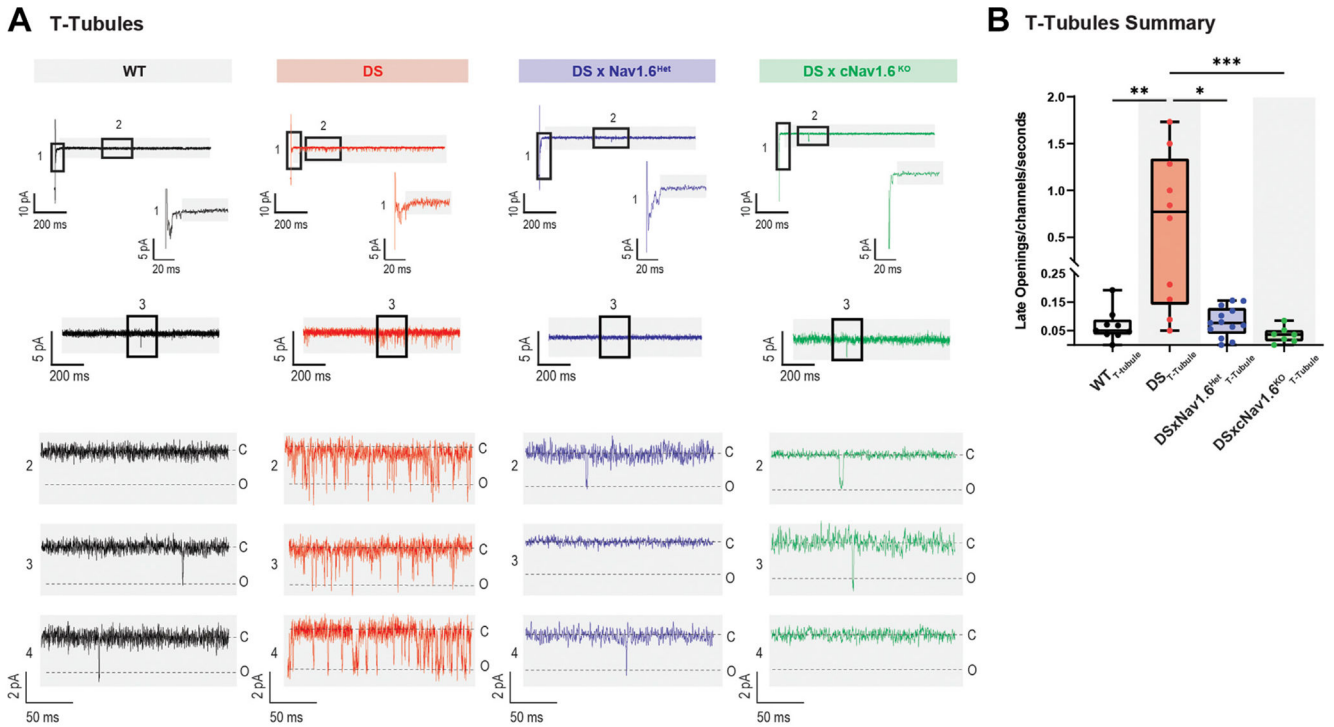
FIGURE 4. Na$_V$1.6 Contributes to Increased Late Na$_V$ Activity Within the T-Tubules of DS Cardiomyocytes

(A) Representative scanning ion conductance microscopy-guided "smart" patch I_{Na} recordings from T-tubule openings. (B) Summary data of late activity at the T-tubules (WT: n = 9 cells, 5 mice; DS: n = 13 cells, 7 mice; DSxNa$_V$1.6^{Het}: n = 13 cells, 5 mice; DSxcNa$_V$1.6^{KO}: n = 7 cells, 4 mice). *$q < 0.05$, **$q < 0.01$, ***$q < 0.001$ relative to DS. Abbreviations as in Figures 1 to 3.

A Na$_v$1.6 Western Blots

WT   DS

250 kDa — ___ Na$_v$1.6

WT   DS

37 kDa — ___ GAPDH
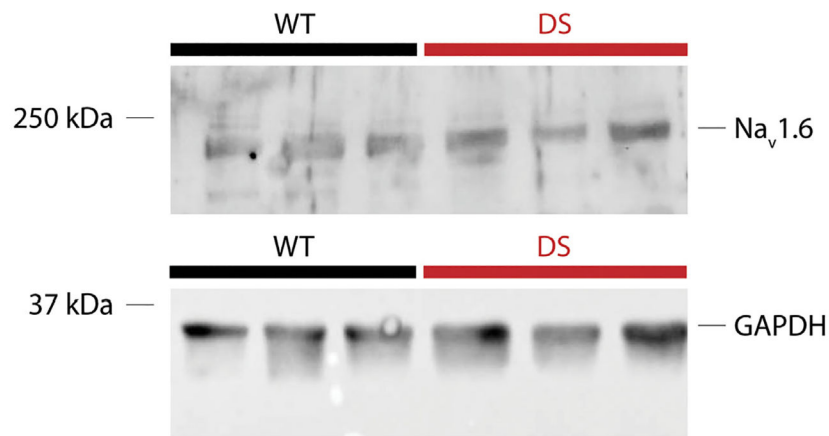
B Western Blot Summary
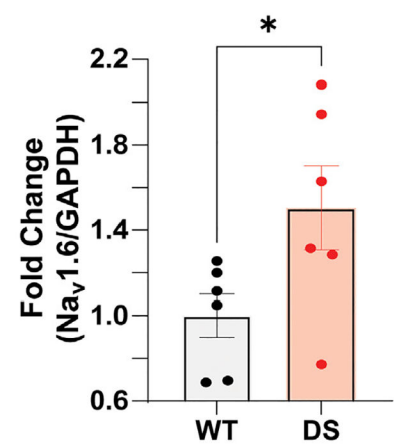
FIGURE 5. Increased Na$_V$1.6 Expression in DS Hearts
(A) Representative Western blots of Na$_V$1.6 and GAPDH (loading control) in WT and DS hearts. (B) Summary Western blot data (n = 6). *P < 0.05 relative to DS. GAPDH = glyceraldehyde 3-phosphate dehydrogenase; other abbreviations as in Figures 1 to 3.
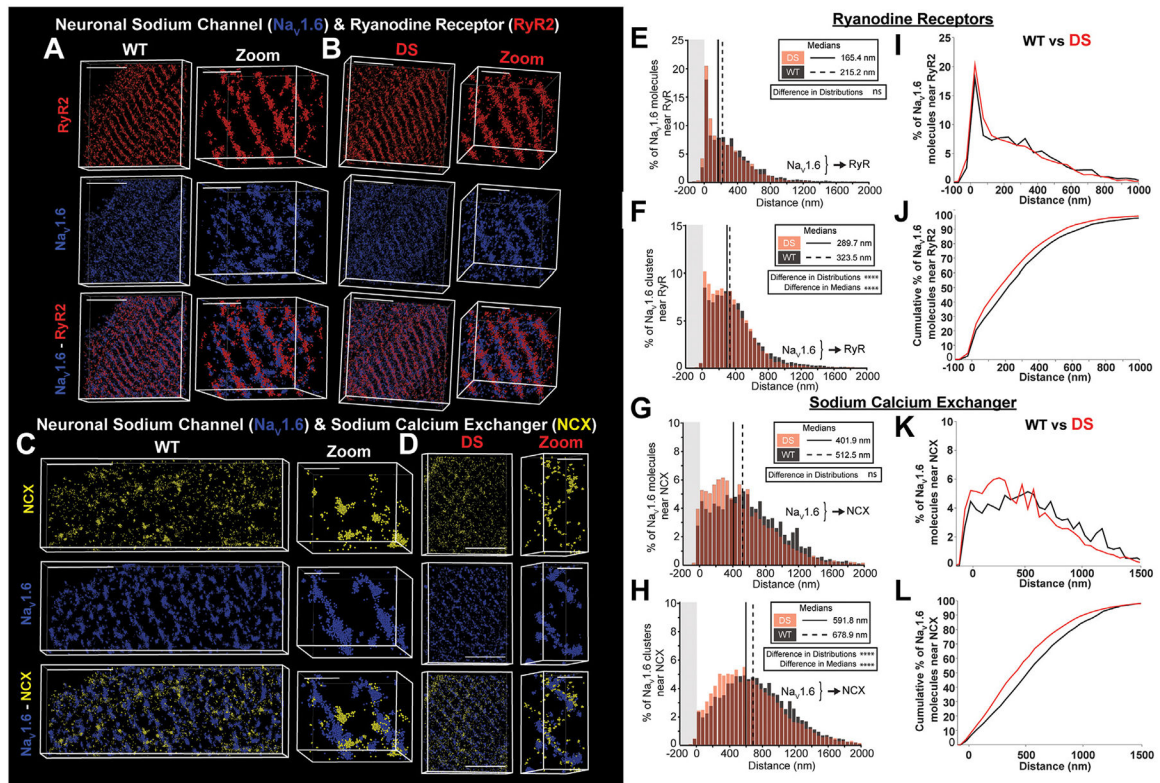
FIGURE 6. STORM Reveals Enhanced Na$_V$1.6 Clustering in Close Proximity to NCX and RyR2 in DS Hearts

Representative stochastic optical reconstruction microscopy (STORM) images from (A and C) WT and (B and D) DS hearts immunolabeled for RyR2 (top, red), Na$_V$1.6 (top and bottom, blue), and NCX (bottom, yellow). Protein distribution measured as percentage of molecules (E: Na$_V$1.6 to RyR2; G: Na$_V$1.6 to NCX) and percentage of clusters (F: Na$_V$1.6 to RyR2; H: Na$_V$1.6 to NCX) (n = 5 images/heart from 3 WT hearts and 3 DS hearts). Differences in distributions and medians were tested with the 2-sample Kolmogorov-Smirnov test and Wilcoxon rank-sum test test, respectively ($P > 0.05$: ns; *P 0.05; **P 0.01; ***P 0.001; ****P 0.0001). (I) Probability distribution and (J) cumulative distribution plots of Na$_V$1.6 clusters relative to RyR2 in WT and DS mice. (K) Probability distribution and (L) cumulative distribution plots of Na$_V$1.6 clusters relative to NCX in WT and DS mice (n = 5 images/heart from 3 WT hearts and 3 DS hearts). Scale bars: (A, left) 7 μm, (A, right) 2 μm, (B, left) 7 μm, (B, right) 2 μm, (C, left) 5 μm, (C, right) 1 μm, (D, left) 6 μm, and (D, right) 1 μm. NCX = sodium/calcium exchanger; other abbreviations as in Figures 1 to 3.
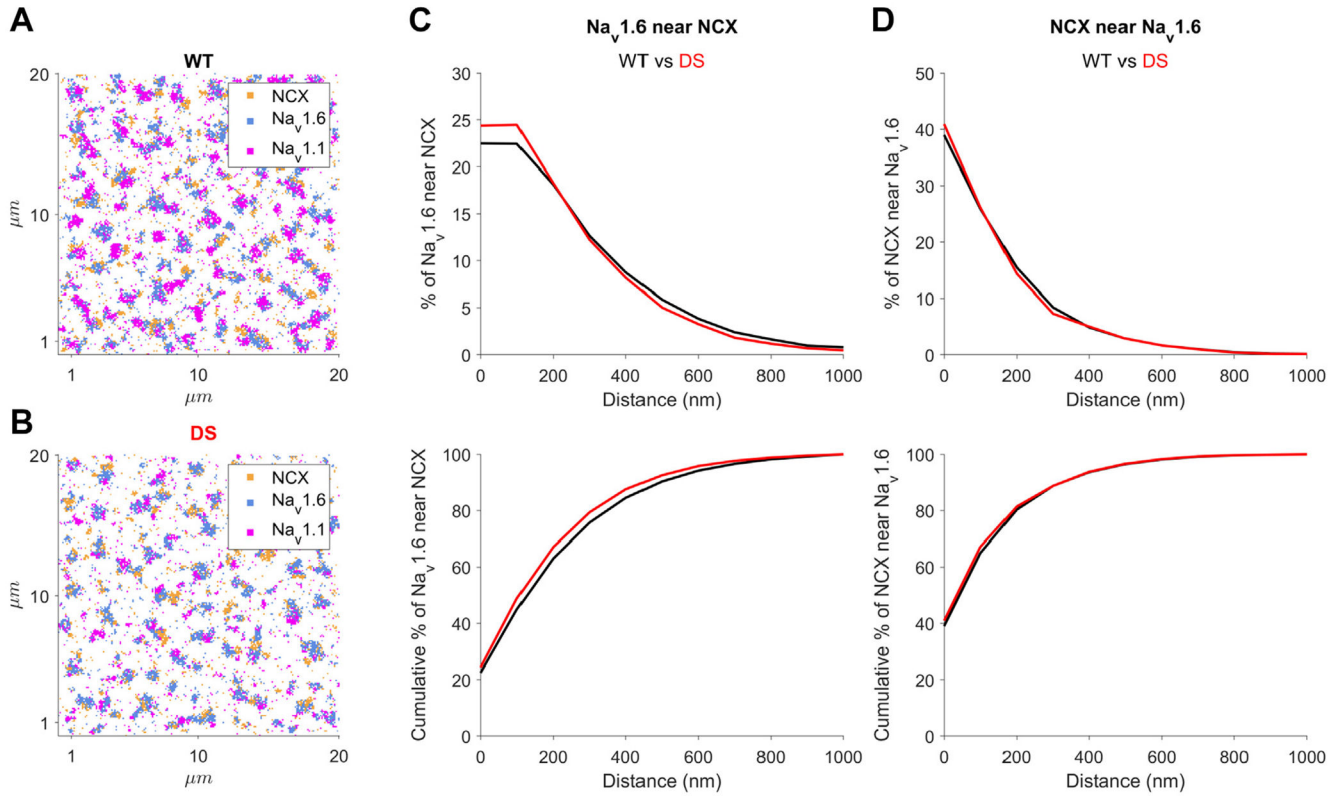
FIGURE 7. Monte Carlo Clustering Simulations Predict That Channel-Channel Interactions Are Sufficient to Explain Enhanced Na$_V$1.6 Clustering in DS

(A) Representative map showing simulated NCX, Na$_V$1.6, and Na$_V$1.1 clustering. (B) Representative map of NCX, Na$_V$1.6, and Na$_V$1.1 clustering in DS. Note the smaller Na$_V$1.1 cluster. (C) Distribution and cumulative distribution plots showing the distribution of Na$_V$1.6 relative to NCX. Reduction in Na$_V$1.1 in DS shifts Na$_V$1.1 distribution to the left (red), without other changes in expression or Na$_V$1.6-NCX interactions. (D) Distribution and cumulative distribution plots showing the distribution of NCX relative to Na$_V$1.6. Reduction in Na$_V$1.1 in DS does not change this distribution. The data shown in C and D are pooled from 5 simulations for each case. Abbreviations as in Figures 1 to 3 and 6.
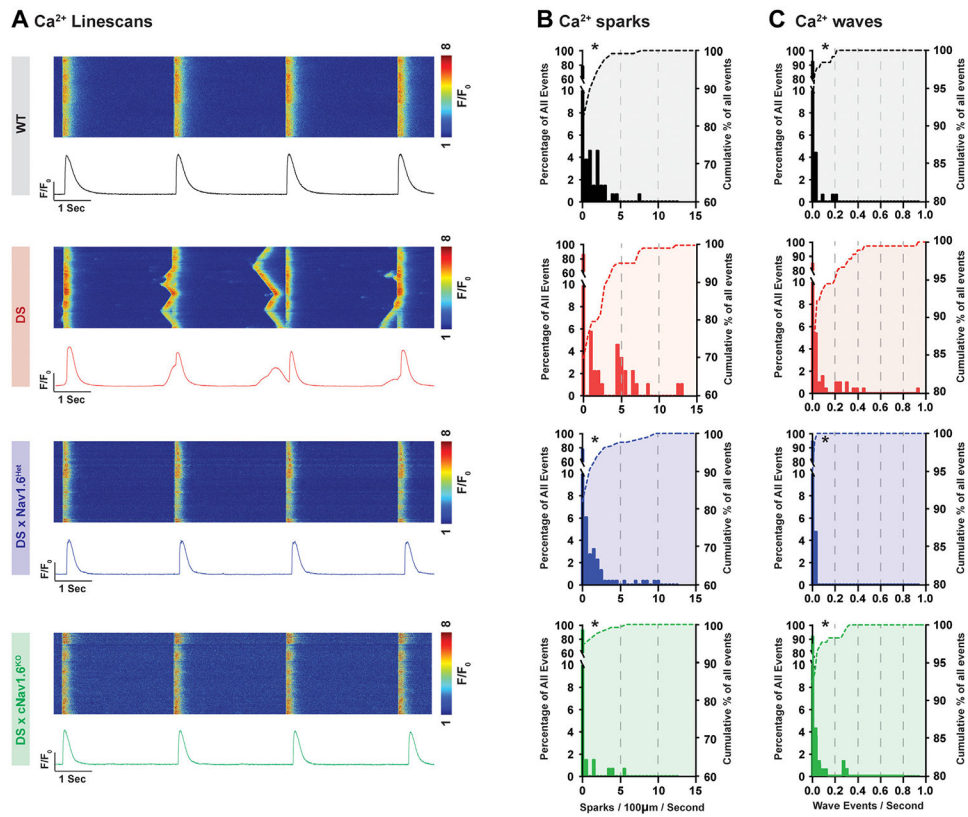
FIGURE 8. Na$_V$1.6 Remodeling Within T-Tubules in DS Contributes to Aberrant Ca^{2+} Release
(A) Representative Ca^{2+} line scans of isolated cardiomyocytes (top) and corresponding Ca^{2+} transients (bottom). (B) Summary of frequency and distribution of Ca^{2+} sparks (WT: n = 121 cells, 7 mice; DS: n = 102 cells, 13 mice; DSxNa$_V$1.6^{Het}: n = 211 cells, 7 mice; DSxcNa$_V$1.6^{KO}: n = 126 cells, 11 mice) and (C) Ca^{2+} waves. WT: n = 123 cells, 7 mice; DS: n = 181 cells, 15 mice; DSxNa$_V$1.6^{Het}: n = 103 cells, 4 mice; DSxcNa$_V$1.6^{KO}: n = 152 cells, 11 mice. *$q < 0.05$ relative to DS. Abbreviations as in Figures 1 to 3.
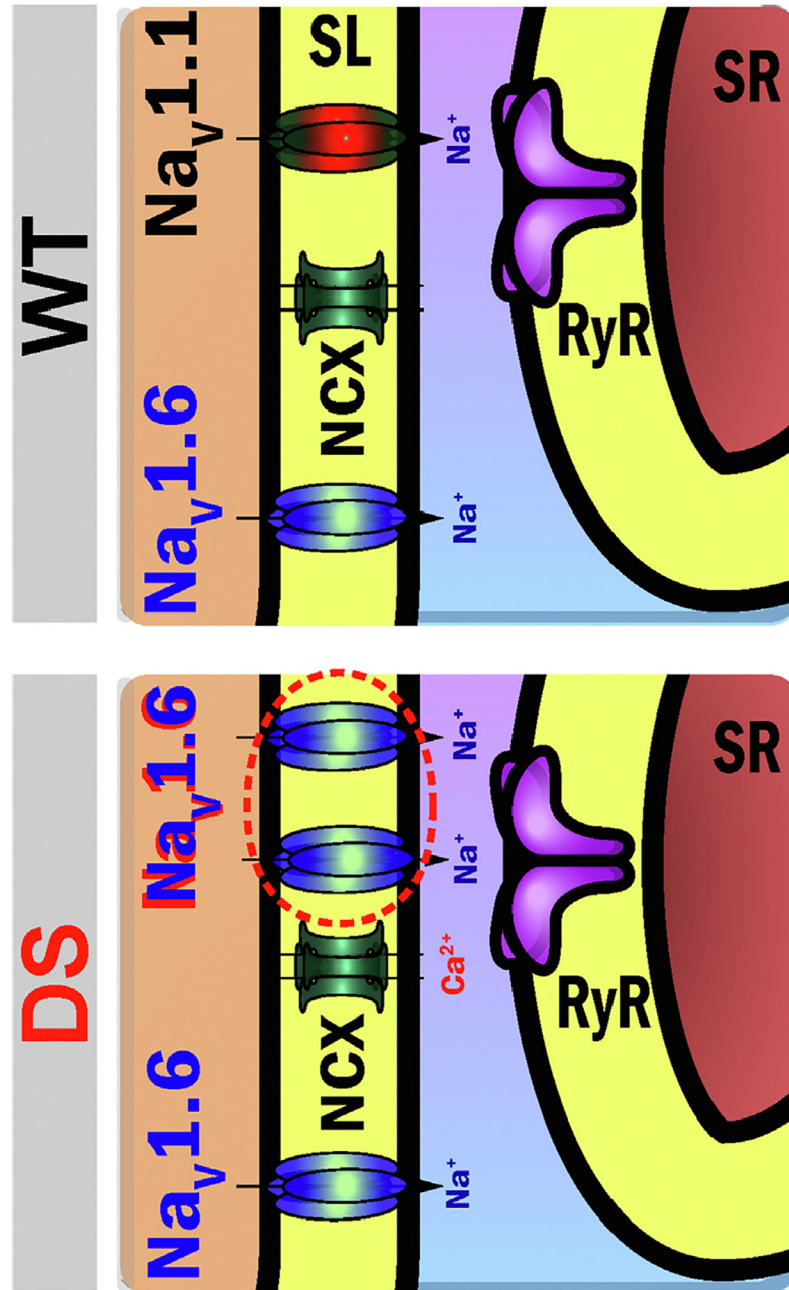
FIGURE 9. Remodeling of Na$_V$s Within the Ca^{2+}-Handling Machinery–Rich Regions of Cardiac T-Tubules in DS Serves as a Substrate for Arrhythmogenic Ca^{2+} Release

Reduction in Na$_V$1.1 evidenced in DS facilitates the repopulation of cardiac sarcolemma (SL) sites typically occupied by Na$_V$1.1 by Na$_V$1.6. The resulting increase in subsarcolemmal Na$^+$ through Na$_V$1.6 promotes Na$^+$/Ca^{2+} exchange and intracellular Ca^{2+} loading, thereby setting a stage for aberrant Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR). Abbreviations as in Figures 1 to 3 and 6.
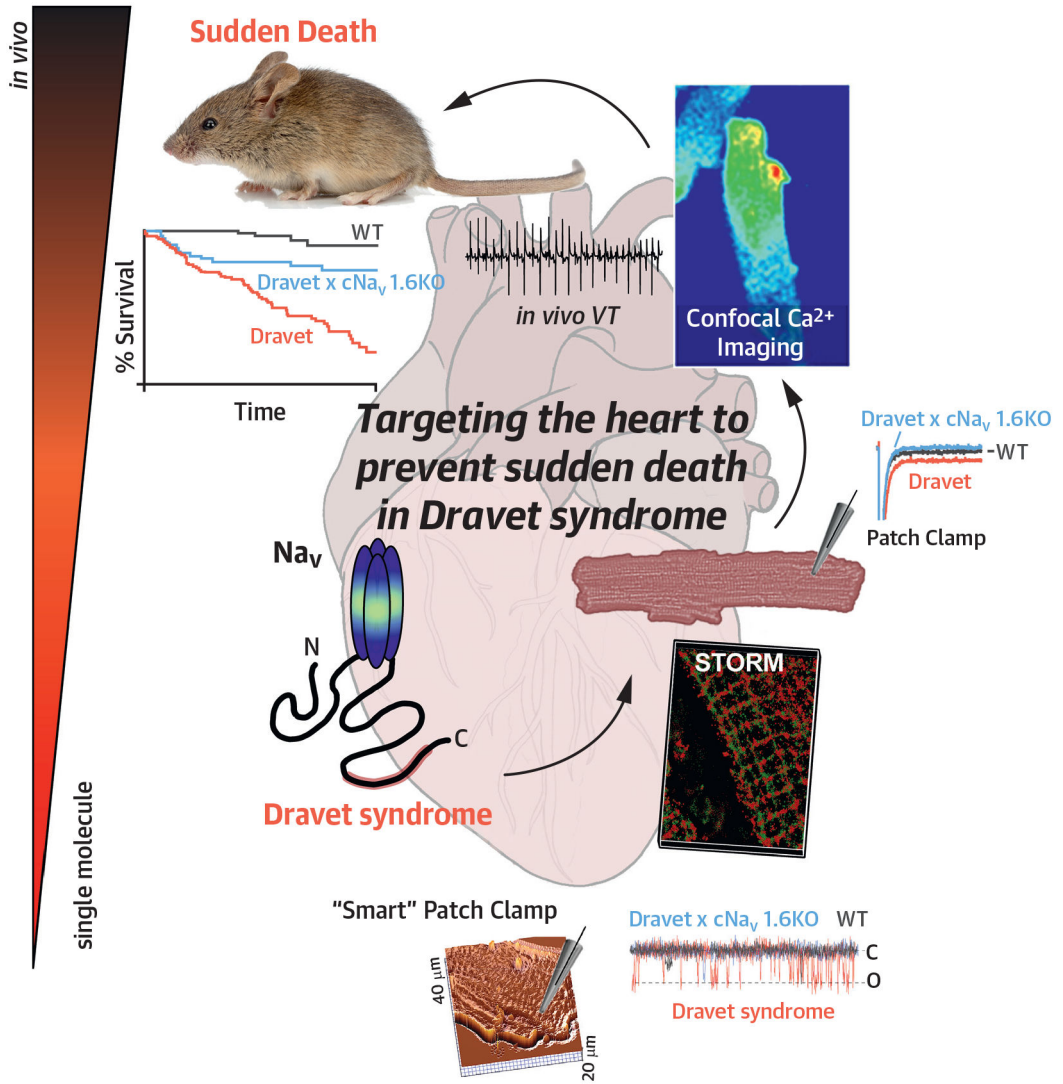
CENTRAL ILLUSTRATION. Targeting the Heart to Prevent Sudden Death in Dravet Syndrome

NA_v = voltage-gated sodium channel; VT = ventricular tachycardia; WT = wild-type.