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Peer Review File

ParSE-seq: A Calibrated Multiplexed Assay to Facilitate the Clinical Classification of Putative Splice-altering Variants

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
In this manuscript, the authors developed a multiplexed minigene-based assay, ParSE-seq, which can assess the splicing consequences for thousands of variants by high-throughput sequencing. Compared to previous splicing assays, ParSE-seq can examine a large number of variants that are located within 250 bp away from the target exon and be calibrated using ClinVar-annotated variants and clinical variant classification in the ACMG scheme. In this analysis, the authors examined over 200 SCN5A variants, and 11 of 42 VUS variants and 29 of 34 variants with conflicting interpretations were reclassified into likely pathogenic or likely benign based on ParSE-seq results. Furthermore, the authors assessed the splice-altering effects of missense and intronic variants by patch clamping experiments.
The authors elaborated on the details in the analytical methods and the manuscript is well written, but it seems unclear along several issues and may need some additions.

Major comments;
1. There are many restrictions on the creation of Minigene, such as restricted enzyme sites, etc. Couldn't it be simpler to use a Gibson assembly, etc.?

2. P5 line.137 290 “clonal genes containing an exon surrounded by and sequence”

3. The process of barcode assignment is too complicated.

4. The bioinformatics pipeline describes Illumina's short-read sequencing part after long-read sequencing using Pacbio and splicing through it.

5. However, it is target exon sequence extraction and the construct design part before long read sequencing automated or manually?

6. For the assembly process, the authors linked each barcode to the synthesized sequence using long read sequencing of the plasmid pool. In general, long reads from third generation sequencers have relatively high error rate. Therefore, long read sequencing data requires QC steps, which is different from short read sequencing data. Please provide the details for QC of long read sequencing, such as the software and statistics.

7. The interpretations of studied variants are classified into three categories; abnormal with deltaPSI_norm < -50%, normal as those with deltaPSI_norm > -20%, and indeterminant as others. How did the authors determine these thresholds? If no criteria

or reference, then multiple thresholds should be set and the validity of these thresholds should be examined.

8. The authors assessed the concordance between ParSE-seq scores and spliceAI. However, some other in silico predictors have been recently developed such as SpliceVault and AbSplice. Please add the comparison and verification between ParSE-seq results and the latest methods.
e.g. https://academic.oup.com/nar/article/50/16/9115/6673120?login=true
Splice AI is far from PERFECT.

8. For the splicing effects of missense variants, the authors picked up 2 missense variants and compared peak current density between these variants and wild type using cDNA-based electrophysiological assay. This result suggests that the patch clamping experiments using cDNA could not detect the effect of these missense variants on SCN5A function, whereas ParSE-seq results indicate these variants showing disrupted splicing. However, the interpretations of these variants are uncertain significance in ClinVar, and whether these variants affect SCN5A function through the disrupted splicing remains unknown. I think that, in order to claim the variant pathogenicity, the authors should consider additional assessment to connect the disrupted splicing of these missense variants with SCN5A function.

9. In addition to the question above, why did the authors use HEK293 cells, not iPSC-CM, to perform patch clamping experiments? If it is not a matter of transfection efficiency in iPSC-CMs, the authors should confirm whether similar results can be obtained using iPSC-CMs.

Minor comments;
1. “Figure 1E” in line 114 at page 5 should be “Figure 1F”.

2. Please add a little more detail in Figure 6A legend, such as workflow as well as colors description.

3. Some of SCN5A gene name are not italicized.

Reviewer #2:
Remarks to the Author:
In this study, O’Neill et al described a ParSE-seq approach to access simultaneously the splicing impact of a large number of variants of SCN5A. The ParSE-seq was

perfomed in two cell lines, HEK293 and iPSC-Cms and validated on variants tagged in clinVAr as pathogenic or benign. In a second step, this approach was used to reinterpret VUS or CI annotated variants. The authors reclassified 40 VUS/CI as LP/LB. This study represent a very important and useful work.

However, I have some remarks:

Majors:
-Selection of variants: did all the variants included in this study came from clinVar? The sentence line 114-115 seemed to suggest that only the LP/P and LB/B variants were from clinvar while all variants referenced in Table S3 have clinVAr ID. Conversely, some variants listed in table SIV and SV had no clinVar ID. Please clarify this point in the text. I suggested to the authors to indicate in the beginning of the result section both the origin and the number of variant finally selected in all categories.

-Figure 2E: The inclusion of the WT exon 6B was very low in both cell lines. Consequently, a low sensitivity of the test for the variants located near this exon could be hypothesized. The exon 6B is an alternative exon and maybe this is related. This point should be discussed in the text (e.g. in the section “limitation of the test”).

-ACMG Assay calibration: this part is very interesting and rarely present in publication. However, I think that the performances of the test could be overestimated due to the control variants selections. Indeed, most of the P/LP variants selected affected the AG/GT invariants dinucleotide. It is known that Minigene assay is particularly sensitive for this class of variant. However, functional assay was used primarily for variants affecting other positions of splice site than AG/GT. A discussion about the performance of the test should be added in the text.

-Cryptic splicing assay of missense variant :
-A supplemental table indicating the REVEL and structural penetrance results should be added
-The authors studded 2 missense variants using cell expression experiment (p.T1131I and p.A1407G). How these variants were selected? Both variants had no impact on the protein function but led to splice abnormality. It seemed to be interesting to compare these results with the specific missense/splice in silico predictions.
-The comparison of missense impact predictors and ParSe-Seq was questionable. These different approaches were created to detect different effects.
- In the text, the authors seemed suggested that ParSe-

Seq could be a best approach to cDNA-based assay for missense variant interpretation. Missense variants could impact the protein function, the splicing or both. ParSe-Seq and cDNA-based assay could be complementary for missense variant and maybe in silico tools could guide the choice of functional test ? This point should be discussed in the text.

- No indication about the type of splicing impact (e.g. exon skipping, activation of cryptic splice site) was given in the result section. This point could be important for clinical interpretation notably if the variant led to inframe impact. In this study, the authors seemed to be considered that all splicing impact was necessarily pathological (regardless the inframe or out of frame impact). This point should be indicated in text and argued (maybe no inframe insertion in SCN5A is known as benign)

-Recapitulating Splice Effect at the endogenous locus : as the splicing impact was confirmed using CRISPR for only one variant, this part did not seem to be essential. If the authors want to keep it, the choice of the variant should be argued. Moreover, in the discussion section, the place of CRISPR assay comparing to in silico/ ParSe-Seq should be discussed.

Minor
-Line 386-401 (Methodology Overview and feasibility) : this paragraph largely overlapped with tee Method section without presenting any results. This paragraph should be removed from the text and the Figure 1 should be introduced throughout the Method section. The same remark could be made for the following paragraph (Assay implementation). The results started to be presented only from line 411.
-In supplementary Table IV and V, the column “ClinVar Clinical Significance” should be added for easier reading
-The figure 2A largely overlapped with the Figure SI. These two figures should be merged

Reviewer #3:
Remarks to the Author:
O’Neill et al study splicing effects of mutations in the arrhythmia-associated SCN5A gene using a multiplexed minigene reporter assay. They relate the measured splicing effects to own electrophysiological measurements and to clinical evidence relating mutations to disease outcome in Brugada syndrome.

To systematically test for splicing changes within disease-associated genes, the authors introduce ParSE-seq, by which they study how the splicing of almost 20 exons is affected by exonic and nearby intronic mutations. For the SCN5A gene, they report a strong correlation of measured splicing effects with in silico predictions and with known clinical effects of the variants. In a more exploratory part, they use their method to classify variants of unknown clinical significance and functionally test three selected mutants.

While the initial screening results are interesting and convincing, the relevance of the validation part remains much less clear (see below). Furthermore, the study remains mostly descriptive with little focus on molecular mechanisms. Finally, the presentation of the results should be improved, as the rationale and description often remain vague.

1. The reporter constructs were built based on separated individual exons of SCN5A, embedded in an exogenous sequence context. Therefore, splicing outcomes in the minigene reporter likely deviate strongly from alternative splicing patterns of the endogenous SCN5A pre-mRNA. Some potentially relevant splicing outcomes (like intron retention) likely cannot be analyzed at all in a meaningful way.

- The authors should clarify how their results link to normal splicing behavior of SCN5A exons? Is the PSI of the reporter constructs similar to the PSI of the endogenous exons? Why is no exon included more than 75% (Fig. 2E)?

- Why Rat IR exons were chosen as flanking sequences? How do these sequences, e.g., possible ESE/ESS sites within them, affect splicing behavior compared to the endogenous gene?

- The authors should more clearly state which splicing fates are covered by their mingene and which ones cannot be studied (likely the case for intron retention, skipping of multiple exons at a time, or mutually exclusive exon inclusion which frequently contribute to pathological outcomes as well)

2. From just reading the main text and figures, many aspects of the minigene assay and results are not clear. The authors should briefly explain the following aspects in the main text without the necessity that readers need to check this in the Supplement/Methods or previous work.

- Which regions/exons in SCN5A were studied? Why was only a subset of exons studied in the end?

- How were mutations chosen and how was the mutagenesis performed?

- The authors should specifiy in the text the kinds of "variants" they are investigating (e.g. SNPs, InDels, etc.) and show how frequently these variants occur in patients.

- Please briefly explain dPSI_norm in the main text. In what sense is this normalized?

- Please also briefly explain some aspects of the reporter system in the main text: another plasmid/vector system, pAG424, is mentioned and described to be part of the plasmids used for splicing analysis of SCN5A. However, it is unclear from the methods and/or (supplementary) figures how the two vectors pET01 and pAG424 are related.

3. The dPSI is known to depend on the starting PSI (Baeza-Centurion et al., 2019; Braun et al., 2018).

- Is this finding true in the present dataset as well? (How) do the conclusions change if the starting PSI is taken into account?

- Why do the authors dPSI_norm > -20% assumed to be ‘normal’? In the methods part, the dPSI_norm formula probably lacks a factor of 100.

4. Functional study and SpliceAI: The authors find an impressive concordance of SpliceAI predictions and their experimental findings.

- To allow the readers to better judge these results, they should describe in more detail what the (aggregated) SpliceAI scores actually predict in terms of molecular splicing events and what actually happens at the molecular (junction) level in their assays (beyond changes in the PSI metric that combines many different molecular changes).

- Since the authors want to focus also on splice variants with a disruption that is not at the canonical 2-bp splice-site motifs, wouldn’t it be more reasonable to show Suppl. Fig VIII in the main figures instead of Fig4B? While in Fig4B R^2 is higher than in SFigVIII, the majority of data points seem to accumulate at the extremes, which not only makes Fig4B look more noisy, but also looking at SFigVIII, these data points are mostly disruptions of the canonical splice sites, which is somewhat trivial.

- How do the authors comment on the outliers in Fig4B and SfigVIII (lower left and upper right points)? The discrepancy is mainly due to limitations in SpliceAI algorithm or ParSE-seq assay?

- The details of LOESS fitting, e.g., brief method introduction, key parameters, etc., needs to be added in the method section.

5. In the sections “ACMG assay calibration” and “variant reclassification”, the authors seem to compare their splicing results to previously reported variant association with clinical phenotypes.

- While the comparison to variants of known clinical significance seems impressive, the authors should explain in more detail the used ClinVar classification. Is it really expected that each splicing defect has a strong impact on protein function? To justify this assumption that also underlies the subsequent variant reclassification, the authors should provide more molecular details about splicing alterations in the studied variants and should discuss their expected effect on protein function and expression level.

- How robust are the reclassification results? Can the prediction power be tested computationally, e.g., cross validation, or by other experimental/clinical data?

6. The rationale underlying Fig. 6 should be explained in more detail:
What kind of splicing effects do the authors specifically think of that are not captured by the cDNA assay? The authors perform patch-clamp assays and show that the two studied variants have normal electrophysiological function, Then, they employ in silico analyses (of unclear relevance) to conclude that these are “splice-alterating variants for which cDNA-based assays of protein function yield incorrect conclusions”. In its present form, this part of the paper is not convincing.

7. In Fig. 7, the authors study a single mutant in the endogenous SCN5A gene in iPSC-CM using genome editing. They show a strong effect in patch-clamp measurements, but only a very weak accumulation of the predicted variant splice isoform in the heterozygous iPSCs – how do these observations match together? The authors should also clarify how common this mutation is in patients to allow the readers to better judge the significance of their result. Similarly, the prevalence should be provided for all disease-associated mutations considered in this work.

8. Representations of the text and figures need to be further improved. Often the figure legend only repeats what is stated in the text without providing necessary details on what is shown and how these figures/results were obtained. Further examples below:

- The Abstract contains a lot of technical terminology and should be formulated in more general terms for a journal with a broad readership like Nature Communications.

- Fig. 1C, for example, lacks a proper legend

- Figure 6: The small graph on the bottom right of Fig6A requires axis labels and some kind of description / attribution in the figure legend. It would also be interesting to e.g. color-mark the data points in Fig6E that correspond to the two SNPs analyzed in C and D, if they are included here.

Reviewer #4:
Remarks to the Author:
The manuscript entitled “ParSE-seq: A Calibrated Multiplexed Assay to Facilitate the Clinical Classification of Putative Splice-altering Variants” report a very powerful use of biotechnology to understanding the consequences of genetic muations on the function, in this ase, of ion channel. But of course it can be applied to any other functional proteins. In particular its usfulness is in regards to mutations found in splice-altering variants, that are usually neglected. Due to my background, I limited my revision to the electrophysiology section of the study, for which I have few observations.

Paragraph “Cryptic spliing effects of missense variants”, lines 524-526. The Authors stated that the two VUS had near-normal electrophysiologic function, but they limited their observation to the peak current density. I ask to inverstigate also other electrophysiological propoerties, as it is known that the kinetics of the channels may be affected by mutations, and the voltage dependece as well. Working with authomated patch-clamp may be useful for information coming from a simple Current-voltage relationship protocol (form which, it is possible to have at leat the activation curve of the channel, and also evaluate the kinetic of the fast inativation). I am sure that the Authors are aware that the Nanion Syncropatch can also be implemented with protocol for the study of the availability curve.

In this sense, I also ask to have example of typical current traces of the NaV1.5 p.T1131I and p:A1407G in Figure 6.

Figure 7, panel F, G and H. Comparing the current amplitude of the traces in panel F with the Current-Voltage relationship (IV) in panel H (and doing the same for the Panel G), it emerged that the capacitane of the cells patched should be about 60 pF and 83 pF, respectively. This is quite uncommon dealing with hiPSCs-CM, where the capacitance is ususally 30-40 pF (see for references PMID 27672365 and 23029342). Would it be

possible that instead of single cells, small group of cells was patched? Moreover, looking on the IV reported in panel H, the WT channels peaks at -30 mV (uncommon, it is known to peak at -20 mV). This result could be possibly due to a poor compensation since the current measured are extremely large (see panel F, despite in in this case the compensation is good), and maybe the capacitance large as well. On the otherhand, considering panel G, in this case the traces are not well compensated and the voltage is not well controlled, despite in the IV peaks at -20 mV. I therefore suggest to check the experiments lowering the sodium concentration in the extracellular solution (here is 50 mM for the manual patch clamp, the suggestion is to decrease at 20 mM).

Reviewer #1

In this manuscript, the authors developed a multiplexed minigene-based assay, ParSE-seq, which can assess the splicing consequences for thousands of variants by high-throughput sequencing. Compared to previous splicing assays, ParSE-seq can examine a large number of variants that are located within 250 bp away from the target exon and be calibrated using ClinVar-annotated variants and clinical variant classification in the ACMG scheme. In this analysis, the authors examined over 200 SCN5A variants, and 11 of 42 VUS variants and 29 of 34 variants with conflicting interpretations were reclassified into likely pathogenic or likely benign based on ParSE-seq results. Furthermore, the authors assessed the splice-altering effects of missense and intronic variants by patch clamping experiments.
The authors elaborated on the details in the analytical methods and the manuscript is well written, but it seems unclear along several issues and may need some additions.

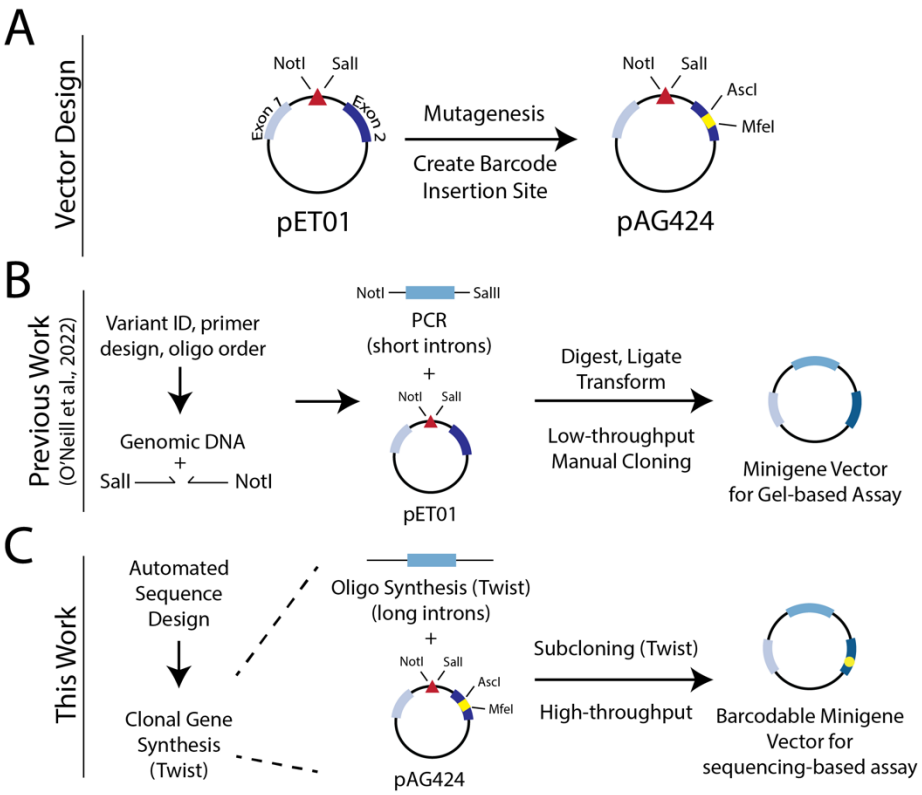
Comment 1: There are many restrictions on the creation of Minigene, such as restricted enzyme sites, etc. Couldn't it be simpler to use a Gibson assembly, etc.?

Response: We previously described a minigene cloning strategy which used standard cloning with restriction digestion that was time-intensive and low-throughput (O'Neill et al, *Circ Genom Precis Med*, **2022**, PMID: 36197721). However, the current ParSE-seq assay relies on an alternate method, Twist clonal gene synthesis technology. In this method an oligonucleotide insert is synthesized and added by Twist Biosciences to the minigene plasmid without restriction digestion. This rapid plasmid generation pipeline enabled the high-throughput experiments and should allow easier adoption by future labs.

We did use restriction digestion for one final cloning step on the plasmid pool, to insert barcodes into the plasmids. In the future, a completely non-restriction based method such as Gibson assembly could be used for this step as well. We have edited the text to clarify our approach:

"290 "clonal genes" containing an exon surrounded by 100-250 bp of intronic sequence (mean = 230) on each side were directly synthesized, inserted into pAG424, and sequence verified by Twist Biosciences (South San Francisco, CA). This rapid Twist clonal gene synthesis method was a modification of our previously described low-throughput, restriction-enzyme based minigene synthesis[14]."

We also now provide a supplemental figure outlining the cloning strategy of the current approach compared to our previous low-throughput minigene strategy:



Supplemental Figure I. Schematic of vector design and cloning overview.
A) pET01 is an established minigene vector containing a MCS between rat insulin exon 1 and 2 and flanking intronic sequences (MoBiTec GmbH). PCR-mutagenesis was used to create a new restriction site in rat insulin exon 2 to allow downstream insertion of a barcode.
B) Schematic of previously described workflow for manual minigene assays using gel-based quantification and manual cloning (PMID: 36197721).
C) Schematic of current approach for high-throughput sequencing-based quantification of splicing. pAG424 was sent to Twist Biosciences, and non-restriction-based cloning of chemically synthesized oligonucleotides was performed.

Comment 2: P5 line.137 290 “clonal genes containing an exon surrounded by and sequence”

Response: We have updated the text which now reads:

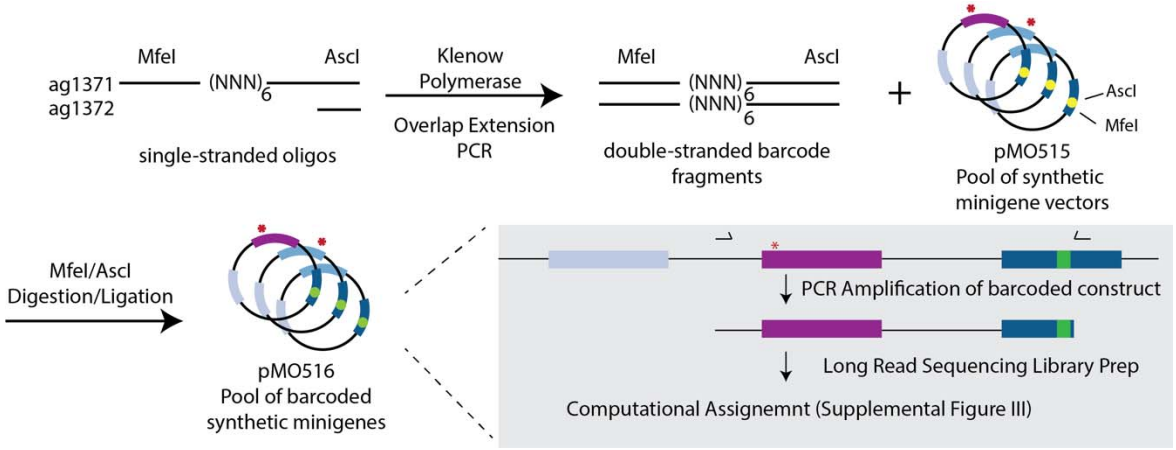
“290 "clonal genes” containing an exon surrounded by 100-250 bp (mean = 230) of intronic sequence”

Comment 3: The process of barcode assignment is too complicated.

Response: We agree that the barcoding process is cumbersome; however, we are constrained by current laboratory techniques for pooled, multiplexed assays. To clarify our barcode assignment process, we have added a new Supplemental Figure II which visualizes the respective experimental steps and workflow. Please see Supplemental Figure III for an additional schematic of the computational workflow. All code to analyze the barcodes and quantify splicing impact is available on GitHub (https://github.com/GlazerLab/ParSE-seq).

We have updated the methods to read:

“We digested the plasmid pool pMO515 with AscI (NEB) and MfeI (NEB) followed by incubation with Calf Intestinal Phosphatase (NEB). An insert containing random 18-mer barcodes was produced (Supplemental Figure II and as previously described (PMID: 37162834)). Briefly, ag1371 and ag1372 were annealed, followed by extension to make fully double stranded DNA using Klenow polymerase (NEB)[37]. Due to its small size, the double stranded DNA was then phenol/chloroform extracted and digested using AscI and MfeI (NEB), and was again purified by phenol/chloroform extraction. The pool of minigene plasmids was also digested with AscI and MfeI and cleaned by gel extraction (QIAGEN). The digested vector pool and barcode inserts were ligated using T4 ligase (NEB).”



Supplemental Figure II. Barcode cloning schematic. Molecular barcodes were created from two single-stranded oligonucleotides, which were annealed and underwent PCR overlap extension. Following purification, oligonucleotides were digested and ligated to a digested pool of minigene vectors (pMO515). Multiple dilutions of barcodes were tested to arrive at a barcoded pool (pMO516), with each unique vector covered by a median of 11 barcodes. Experimentally, barcode assignment was completed with PCR amplicons from this pool. Long-read sequencing libraries were prepared from these amplicons, after which computational analyses presented in the methods, GitHub, and Supplemental Figure III enabled unique assignments.

Comment 4: The bioinformatics pipeline describes Illumina's short-read sequencing part after long-read sequencing using Pacbio and splicing through it. However, it is target exon sequence extraction and the construct design part before long read sequencing automated or manually?

Response: We performed two major tasks to analyze our sequencing data: assembly (long read sequencing linking the barcode to the construct/mutation) and the assay (transfecting the library into cells and short read targeted RNA-seq to determine the percent spliced in for each barcode; Figure 1B). First in the methods we present the assembly computational processing. This was performed in an automated manner using a series of Unix “grep” (search) commands to count the number of sequencing reads associated with each barcode and each target insert. Second in the methods we present the processing of the short-read data that allowed us to determine the splicing outcomes of each barcode. This analysis was also performed in an automated manner using Unix scripts. We present the long read assembly processing first and short read splicing analysis second, because the assembly analysis can be performed

independently, but the short-read splicing analysis requires the results of the assembly data (the link between barcodes and intron:exon:intron sequences/mutations).

We have updated the results to state:

“We performed two major tasks to analyze our sequencing data: first, assembly (long-read sequencing linking the barcode to the construct/mutation) and the assay (inserting the library into cells and, second, short-read targeted RNA-seq to determine the percent spliced in for each barcode; Figure 1B).”

Comment 5: For the assembly process, the authors linked each barcode to the synthesized sequence using long read sequencing of the plasmid pool. In general, long reads from third generation sequencers have relatively high error rate. Therefore, long read sequencing data requires QC steps, which is different from short read sequencing data. Please provide the details for QC of long read sequencing, such as the software and statistics.

Response: To mitigate the higher error rate of PacBio sequencing, we only analyzed circular consensus reads. This process (a common method performed by the Maryland Genomics sequencing center) involved circularizing each DNA molecule and repeatedly sequencing it (typically >20 times). The sequencing center used the consensus of the repeated sequencing reads to calculate a CCS read for each molecule. The CCS reads are much more accurate than raw single-pass sequencing reads. During our analysis of the CCS reads, we applied further QC to assign barcodes, by only analyzing CCS reads that completely matched the expected flanking sequences at the barcode site and one of the candidate constructs. We have added the following to the methods:

“We recorded 30 hours of PacBio SMRT cell sequencing. To mitigate sequencing errors in the raw PacBio data, we only analyzed Circular Consensus Sequence (CCS) reads. A total of 4,136,990 CCS reads were obtained as fastq files, with an average size of 1,312 base pairs. The median Q score was 48 across CCS reads. Only reads containing an 18-nucleotide barcode with a perfectly matched 6-bp prefix and 8-bp suffix were retained, using the ‘grep’ function in a Unix bash script. We only included barcodes with at least 50 CCS reads for insert assignments (N = 3,303). To ensure high quality assembly, we also analyzed filtered reads with a perfect match to one of the candidate wildtype or mutant constructs across the entire sequence insert using ‘grep’ in a Unix bash script (mean 636 bps; Supplemental Figure IIB).”

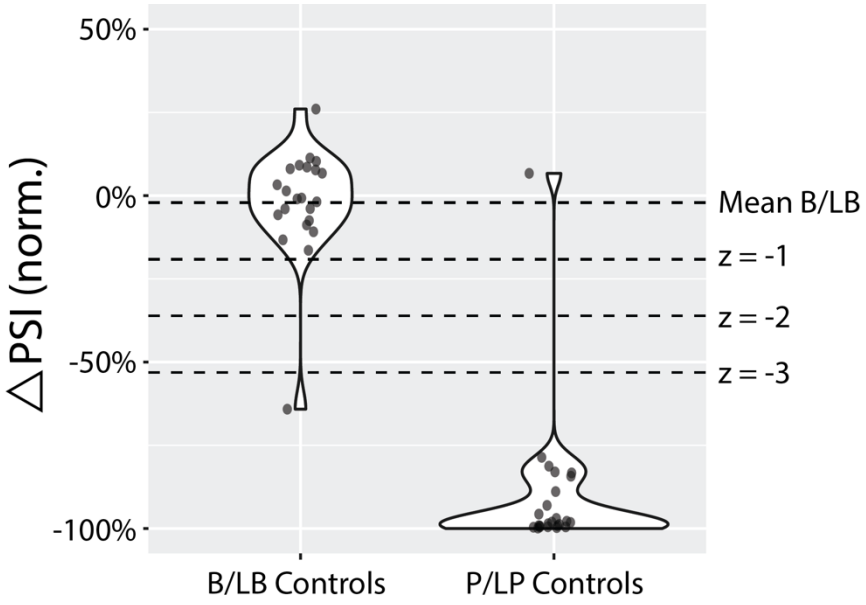
Comment 7: The interpretations of studied variants are classified into three categories; abnormal with deltaPSI_norm < -50%, normal as those with deltaPSI_norm > -20%, and indeterminant as others. How did the authors determine these thresholds? If no criteria or reference, then multiple thresholds should be set and the validity of these thresholds should be examined.

Response: We agree that the choice of a single set of cutoffs is somewhat arbitrary. We now present further explanation and motivation for our primary set of thresholds. As suggested, we also now present a sensitivity analysis using multiple thresholds and show that the primary results of the paper do not substantially change, despite a small number of variants switching categories with altered thresholds.

Our thresholds for abnormal/normal/indeterminant function were derived from the distribution of ΔPSI_norm results on our curated list of Benign and Likely Benign controls, as recommended by

the ClinGen Sequence Variant Interpretation working group (PMID: 31892348). We have updated the methods accordingly:

"Thresholds for normal, abnormal, and indeterminant function were derived from assay performance on Benign (B) and Likely Benign (LB) controls as recommended by the ClinGen Sequence Variant Interpretation working group (PMID: 31892348). We used the mean (-2.1%) and standard deviation (17%) of ΔPSI_norm among B/LB variants and created corresponding z-score thresholds (Supplemental Figure IV). To assign functional outcomes, we considered variants with a z-score < -3 (-53%) to be abnormal, and those with a z score > -1 (-19%) to be normal. These corresponded roughly to -50% and -20%, which we used as our primary cutoffs for generalizability across different genes. We considered the range < -20% and >-50% as indeterminant without additional studies to avoid dichotomization of a continuous score. The benign and pathogenic calibrated strengths of evidence (OddsPath) were robust and consistent across a range of thresholds (Supplemental Table IV)."



Supplemental Figure IV. Calibration of Functional Outcomes. We calculated the ΔPSI_norm of B/LB control variants and derived the mean score and standard deviation. We observe excellent stratification of B/LB and P/LP variants.

In a sensitivity analysis, we instead implemented a variety of various cutoffs. We found that the results for calibration of external controls were robust across the choice of thresholds. We now include this analysis as Supplemental Table IV.

Pathogenic Cutoff	Benign Cutoff	P1	P2path	P2benign	OddsPath_P	OddsPath_B	P Strength	B Strength	Assignable Variants
< -36	> -17	0.532	0.960	0.045	21.12	0.0419	PS3	BS3	179
< -72	> -17	0.543	0.96	0.045	20.16	0.04	PS3	BS3	172
< -36	> -36	0.5	0.96	0.040	24	0.0417	PS3	BS3	201
< -50	**> -20**	**0.532**	**0.960**	**0.045**	**21.12**	**0.0419**	**PS3**	**BS3**	**182**
< -40	> -20	0.532	0.960	0.045	21.12	0.0419	PS3	BS3	183

< -30	> -20	0.532	0.960	0.045	21.12	0.0419	PS3	BS3	183
< -20	> -20	0.532	0.960	0.045	21.12	0.0419	PS3	BS3	184

Supplemental Table IV. Sensitivity analysis of functionally abnormal/normal thresholds for control variants and total variant assignments in the iPSC-CM dataset. See Supplemental File I and methods for derivations of OddsPath priors and posteriors. Indeterminant variants are those with normalized PSI changes that fall between the pathogenic or benign cutoffs. The primary analysis used in the paper is highlighted in bold.

Comment 8: The authors assessed the concordance between ParSE-seq scores and spliceAI. However, some other in silico predictors have been recently developed such as SpliceVault and AbSplice. Please add the comparison and verification between ParSE-seq results and the latest methods. e.g. https://academic.oup.com/nar/article/50/16/9115/6673120?login=true. Splice AI is far from PERFECT.

Response: In addition to our SpliceAI analysis, we now present comparisons between Parse-seq data and two other splicing predictors, AbSplice and Pangolin. We did not include SpliceVault as SpliceVault scores are not calculated individually for each variant. These data are now presented alongside SpliceAI as revised Figure 4 and in Supplemental Figure XII. We agree that the computational predictors are far from perfect. Accordingly, and in response to Reviewer 3 Comment 7, we now present correlations between *in silico* predictions and experimental outcomes for non-canonical splice site variants in the main Figure 4D, and all studied variants as Supplemental Figure XI.

We have updated the methods as:

"We accessed pre-computed computed AbSplice data from https://zenodo.org/records/7871809. (PMID: 37142848) We analyzed ENSG00000183873 and restricted the analysis to splicing results for the specific tissue 'Heart – Left Ventricle'. Pangolin scores were obtained using the command line Pangolin tool with default settings and a .csv of chromosomal locations as input (PMID: 35449021)."

The results section:

"In addition to SpliceAI, we also examined correlation of experimental data with *in silico* predictions from AbSplice (PMID: 37142848) and Pangolin (PMID: 35449021) (scores in Supplemental Table III; 3-way *in silico* correlations presented in Supplemental Figure XI). We examined the correlation of each predictor with ΔPSI_norm for all non-canonical splice site variants in Figures 4D-4F. SpliceAI provided the highest correlation with experimental data (Spearman rho = -0.81), followed by Pangolin (Spearman rho = -0.67) and ABSplice (Spearman rho = -0.61). Correlations between effect predictors and ParSE-seq outcomes for all variants, including canonical splice sites, are presented in Supplemental Figure XII."

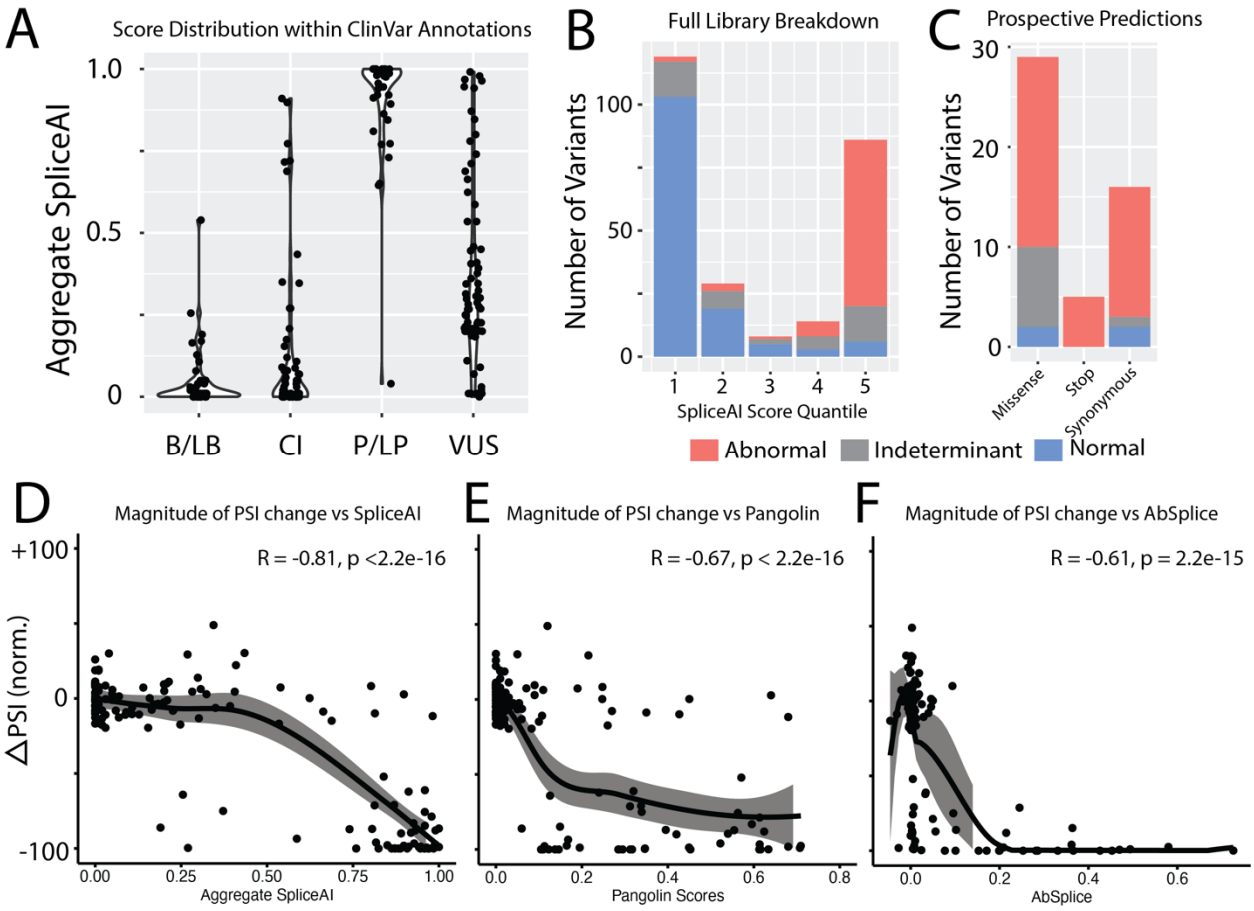
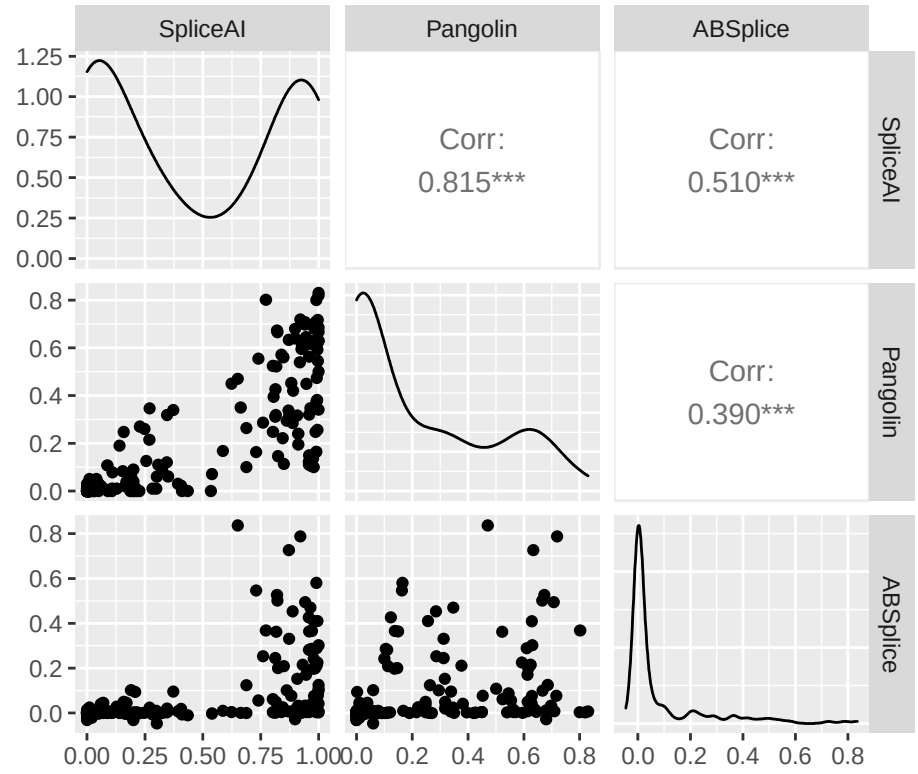
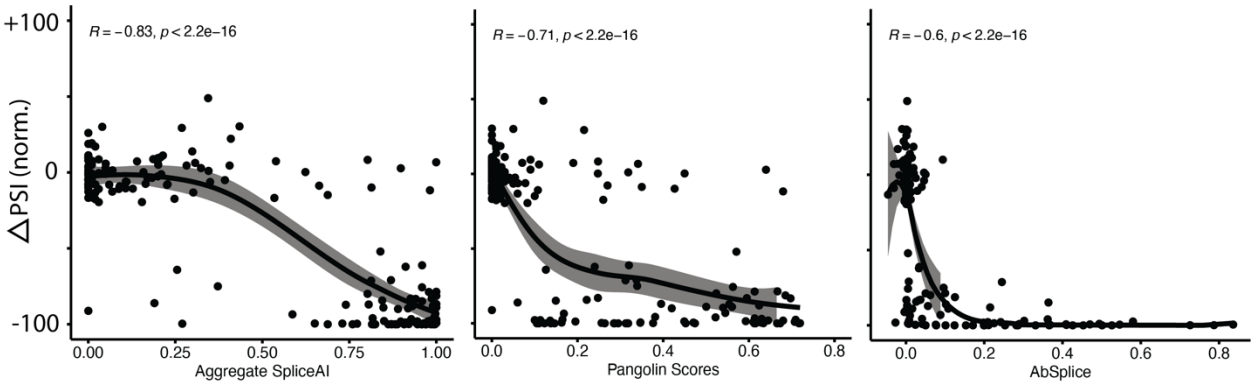


Figure 4. Comparison of experimental data and *in silico* splicing predictors.
A) Aggregate SpliceAI scores for each ClinVar variant class.
B) Distribution of variant effect in ParSE-seq stratified by SpliceAI score quantiles.
C) Results of prospectively identified exonic variants by SpliceAI score >0.8 stratified by mutation type and ParSE-seq outcome.
D-F) Correlation of normalized ΔPSI for non-canonical splice site variants against aggregate SpliceAI scores (D), Pangolin scores (E), and ABSplice scores (F). Confidence interval fit using LOESS (see methods).



Supplemental Figure XI. Spearman correlations of predictions for SpliceAI, Pangolin, and AbSplice for all variants.



Supplemental Figure XII. Spearman rho correlations for SpliceAI, Pangolin, and AbSplice with ΔPSI_norm across all experimental variants, including canonical splice sites.

Comment 9: For the splicing effects of missense variants, the authors picked up 2 missense variants and compared peak current density between these variants and wild type using cDNA-based electrophysiological assay. This result suggests that the patch clamping experiments using cDNA could not detect the effect of these missense variants on SCN5A function, whereas

ParSE-seq results indicate these variants showing disrupted splicing. However, the interpretations of these variants are uncertain significance in ClinVar, and whether these variants affect SCN5A function through the disrupted splicing remains unknown. I think that, in order to claim the variant pathogenicity, the authors should consider additional assessment to connect the disrupted splicing of these missense variants with SCN5A function.

Response: We have further investigated these results in two ways. First, we present other variant electrophysiological properties to further rule out a direct effect on protein function. We found no large differences compared to wildtype *SCN5A* across other relevant features of $Na_V1.5$ protein function, including voltage-dependence of activation and inactivation, or recovery from inactivation. Please see our response to Reviewer 4, Comment 1 for further details.

Second, we have generated and studied an additional CRISPR-edited iPSC-CM line heterozygous for the p.A1407G missense variant (please see updated Figure 6). We now show that in the endogenous context in an iPSC-CM line, this missense variant alters RNA splicing in a pattern consistent with the *in vitro* ParSE-seq experiments. Additionally, we find that peak sodium current is diminished in an iPSC-CM model, but is not perturbed in a cDNA-based HEK cell patch clamp model that does not model splicing.

These new analyses and experiments further strengthen our argument that missense variants can disrupt protein function through a splicing mechanism, and that these effects may not be apparent in cDNA-based functional assays.

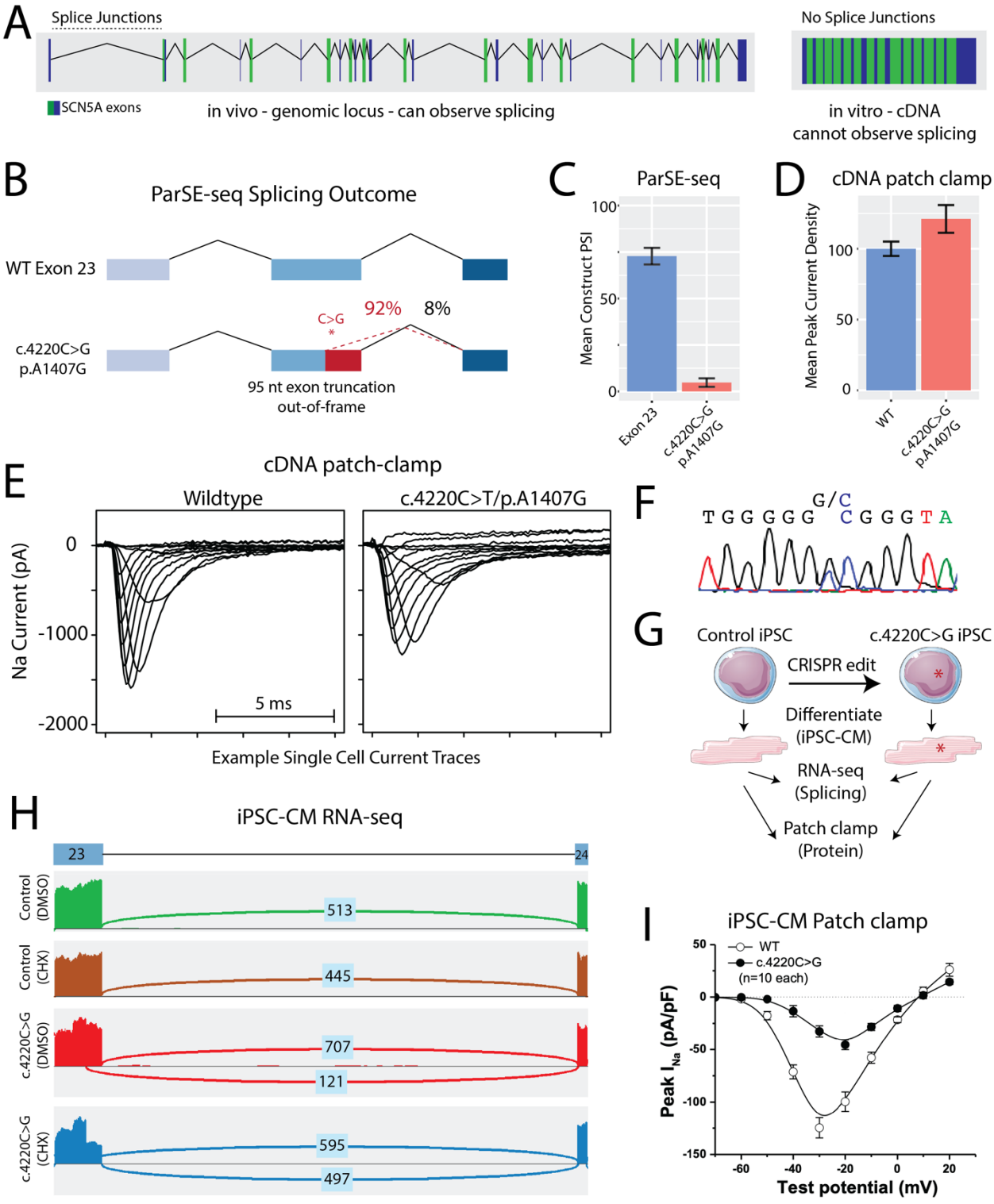


Figure 6. Multi-assay investigation of an *in vitro* splice-altering missense variant.
A) Schematic showing that cDNA-based assays do not account for splice-altering variant effects. Left: Schematic of genomic locus with large intronic sequences; Right: cDNA-based sequence without introns used in many *SCN5A* functional assays. Alternating exons are annotated in green and blue.

B) Molecular analysis of the ParSE-seq assay showed activation of an upstream cryptic splice donor site, resulting in a 31-bp exon truncation.
C) Quantification of mean canonical PSI among reads for the WT exon construct and variant construct. Error bar corresponds to standard error of the mean.
D) Quantification of sodium channel current densities for WT $Na_V1.5$ and variant $Na_V1.5$ using the SyncroPatch automated patch-clamping system (cDNA assay), in stably expressing HEK293 cells. Error bar corresponds to standard error of the mean.
E) Representative single cell sodium current traces for a WT and variant HEK cell. cDNA assessment of this missense variant did not show an effect on protein function when assessed by automated patch-clamping, a system that cannot assess splicing impact.
F) CRISPR editing of a population control induced pluripotent stem cell (iPSC) line was performed to make a heterozygous edit of the line.
G) The WT and heterozygote variant iPSCs were chemically differentiated into cardiomyocytes (iPSC-CMs).
H) Differentiated iPSC-CMs were treated with DMSO or the NMD inhibitor cycloheximide, followed by RNA-isolation and RNA-seq. We observed aberrant splicing consistent with the ParSE-seq molecular event (exon truncation) in the variant, but not WT lines. Notably, treatment with cycloheximide increases the ratio of WT splicing to exon truncation, consistent with NMD degradation of the aberrant transcript.
I) Manual patch-clamp of the WT and variant iPSC-CMs was performed to test the effect of aberrant splicing on protein function. Sodium currents were abrogated in the presence of the variant compared to WT, consistent with haploinsufficiency from loss-of-splicing in panel F.

We have updated the results:

"Cryptic splicing effects of missense variants. Missense variants that disrupt gene function and lead to disease are usually presumed to disrupt protein function. Functional assays of missense variants are often performed using cDNA, which may obscure missense variant effects on splicing (Figure 6A).[63] We hypothesized that some *SCN5A* missense variants may cause Mendelian disorders such as BrS through an aberrant splicing mechanism rather than isolated disruption of protein function. In iPSC-CMs, we recovered determinate data for 48 missense variants; 28 were listed as VUS in ClinVar, and 20 were identified prospectively with high SpliceAI scores (>0.8). Of these, we identified 18 splice-altering missense variants, created by 20 unique splice-altering single nucleotide variants. These variants were distributed throughout the protein, but often clustered in hotspots near exon boundaries. To explore differences in missense variant splicing and protein function in cDNA and endogenous assays, we studied the clinically relevant missense variant c.4220C>G/p.A1407G. This variant was reported in a patient undergoing genetic evaluation for *SCN5A*-related disease in ClinVar and disrupted splicing in the ParSE-seq assay by causing a large out of-frame exon truncation event (Figure 6B-C). We then performed cDNA-based automated patch clamping experiments on this variant using HEK293 cells stably expressing *SCN5A* cDNA (Figure 6C and 6D). The variant had near-normal electrophysiologic function in this assay, with a normalized peak current density of 121.9±9.1% (n=46) of WT (Figure 6C and 6D). In addition to peak current, we also studied additional electrophysiological functions of voltages of half-activation, voltages of half-inactivation, recovery from inactivation, time constant τ of inactivation, and late current (Supplemental Figure XIV and Supplemental Table VII). We did not observe large differences in these parameters, except a minor left-shift of activation for p.A1407G and a right-shift of inactivation for p.A1407G, which are both predicted to cause slight gain-of-function. We also characterized a second missense variant, c.3392C>T/p.T1131I, which disrupted splicing in the ParSE-seq assay but

had near-normal electrophysiological functions in the cDNA assay (Supplemental Figure XIV and Supplemental Table VIII).

Given the discrepancy in *in vitro* assays, we chose to further study the effect on splicing and protein function by introducing the c.4220C>G/p.A1407G variant at the endogenous locus in iPSC-CMs[64]. Using CRISPR-Cas9, we generated a heterozygous edit of c.4220C>G in a population control iPSC line (Figure 6E). Alongside the WT population control, both lines were differentiated into iPSC-CMs using a chemical differentiation method and then studied at the RNA level (RNA-seq) and protein level (patch-clamp; Figure 6F). To avoid confounding by nonsense-mediated decay (NMD) in the endogenous locus of heterozygous iPSC-CMs[65,66], we treated both isogenic lines with the NMD inhibitor cycloheximide (CHX) alongside a vehicle control (DMSO). We performed RNA-seq and observed reads corresponding to the ParSE-seq exon truncation event in the variant line treated with vehicle control (Figure 6H). The aberrant reads were increased after treatment with CHX (consistent with NMD degradation of out-of-frame transcript; Figure 6H). This truncation event was not observed in isogenic control lines treated with either DMSO or CHX (Figure 6H). To test whether variant-induced aberrant splicing affected protein-level function, isogenic pairs of iPSC-CMs were studied by patch clamping to measure sodium channel current (Figures 6I). We observed a decrease in sodium current across a range of voltages in the c.4220C>G line compared to the isogenic control, confirming the hallmark loss-of-function phenotype for *SCN5A*-linked BrS[67,68] (Figure 6I).

Thus, ParSE-seq can help identify a class of missense, splice-altering variants for which cDNA-based assays of protein function yield incorrect conclusions about variant pathogenicity. This result highlights that for missense variants, ParSE-seq can be used to complement traditional cDNA-based assays of protein function."

Comment 10: In addition to the question above, why did the authors use HEK293 cells, not iPSC-CM, to perform patch clamping experiments? If it is not a matter of transfection efficiency in iPSC-CMs, the authors should confirm whether similar results can be obtained using iPSC-CMs.

Response: Heterologous expression experiments (transfection of the gene into a cell line such as HEK293 cells that does not express the gene endogenously) are still the most commonly used method to evaluate the function of ion channel mutations. Although our group performs many of these studies, one of the messages of our paper is that these experiments rely on pre-spliced together cDNA and thus can yield inaccurate results for variants that affect splicing. For this reason, we chose to perform most of the patch clamp experiments in HEK293 cells, to emphasize that the most commonly used cell model/experiment type can yield inaccurate results if splicing effects are not considered.

There are two major reasons we did not do the patch-clamp transfection experiments in iPSC-CMs. 1) HEK293 cells are the most common cell model used to study SCN5A variant function (440 of 524 experiments in a large literature review of *SCN5A* variants that we previously performed, PMID 29728395). 2) iPSC-CMs express endogenous wildtype *SCN5A*, so we cannot simply transfect these cells with variant *SCN5A*. Finally, we note that the effect that we are trying to emphasize (that assays using cDNA cannot capture splicing effects) would also be predicted to hold true regardless of cell type.

Instead of transient expression of iPSC-CMs, we perform CRIPSR editing experiments to edit the wildtype *SCN5A* allele to a variant allele. These experiments are more time-consuming and expensive than heterologous expression experiments. We did perform one of these CRISPR-editing experiments for a variant of particular interest, c.1891-4C>G (updated Supplemental Figure XV). In addition, we now provide data for an additional missense variant p.A1407G/c.4220C>G in CRISPR-edited iPSC-CMs (current main Figure 6). This new experimental work directly highlights the difference of splice-perturbing variants in cDNA vs endogenous contexts.

We have added the following to the text discussion:

"Although CRISPR edited iPSC-CMs are rising in frequency as a cellular model to study variant effects, these experiments are still relatively low-throughput and resource-intensive. Heterologous expression in HEK293 cells is the most commonly used cell model to study *SCN5A* variant function (440 of 524 experiments in a large literature review we performed in 2018 [PMID 29728395])."

Comment 11: "Figure 1E" in line 114 at page 5 should be "Figure 1F".

Response: We have corrected this typo.

Comment 12: Please add a little more detail in Figure 6A legend, such as workflow as well as colors description.

Response: The Figure 6A legend (see Comment 9 above) now reads:

Schematic showing that cDNA-based assays do not account for splice-altering variant effects. Left: Schematic of genomic locus with large intronic sequences; Right: cDNA-based sequence without introns used in many *SCN5A* functional assays. Alternating exons are annotated in green and blue.

Comment 13: Some of SCN5A gene name are not italicized.

Response: We now consistently use '*SCN5A*' when referring to the gene, and '$Na_V1.5$' to refer to the translated protein.

Reviewer #2

In this study, O'Neill et al described a ParSE-seq approach to access simultaneously the splicing impact of a large number of variants of SCN5A. The ParSE-seq was performed in two cell lines, HEK293 and iPSC-CMs and validated on variants tagged in ClinVar as pathogenic or benign. In a second step, this approach was used to reinterpret VUS or CI annotated variants. The authors reclassified 40 VUS/CI as LP/LB. This study represents a very important and useful work.

Comment 1: Selection of variants: did all the variants included in this study came from ClinVar? The sentence line 114-115 seemed to suggest that only the LP/P and LB/B variants were from ClinVar while all variants referenced in Table S3 have ClinVar ID. Conversely, some variants listed in table SIV and SV had no ClinVar ID. Please clarify this point in the text. I suggested to the authors to indicate in the beginning of the result section both the origin and the number of variant finally selected in all categories.

Response: We now include an updated figure panel to better describe the variant selection and overlap among variant categories in the plasmid library.

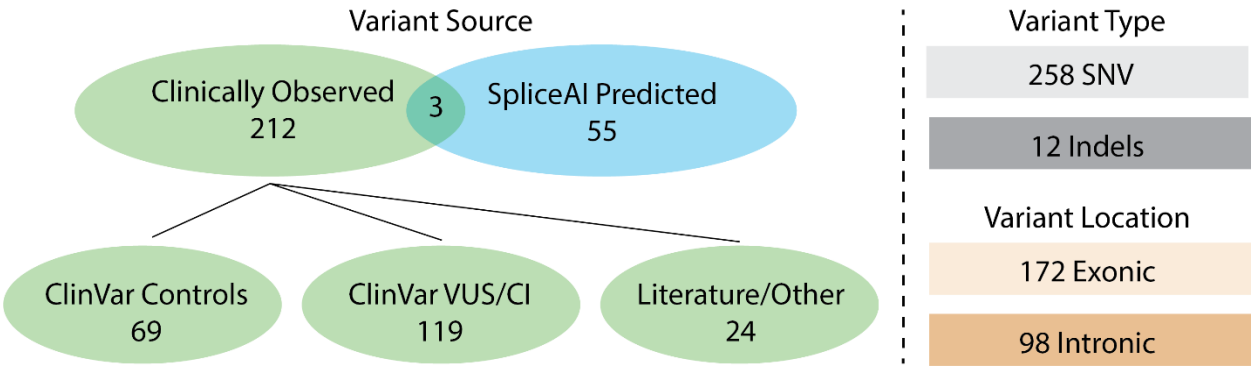


Figure 1F) Variants were collected from two primary sources – clinically observed *SCN5A* variants and prospectively identified *SCN5A* variants with high SpliceAI scores. Variants were mostly single nucleotide variants with some insertion/deletions, and more exonic variants were studied than intronic.

The result section has been updated:

"We studied variants from two groups: 1) exonic and intronic variants clinically observed in individuals; and 2) exonic variants predicted by SpliceAI to disrupt splicing (Figure 1F). Clinically observed variants came from gnomAD, ClinVar, and literature reports (some of which were not present in ClinVar). B/LB and P/LP variants from ClinVar were used to calibrate the assay (see methods; N = 69), after which we applied calibrated functional evidence criteria to facilitate classification of VUS and Conflicting Interpretation Variants (N = 119). These included 258 single nucleotide variants and 12 indels. There were 58 exonic single nucleotide variants with high aggregate SpliceAI scores (>0.80), of which 3 had been observed in ClinVar (2 VUS, 1 Conflicting Interpretation)."

As clarified here, variants without ClinVar IDs in Table S3 and other tables were from literature reports and from variants with high *in silico* predictions of splice-disruption.

Comment 2: Figure 2E: The inclusion of the WT exon 6B was very low in both cell lines. Consequently, a low sensitivity of the test for the variants located near this exon could be hypothesized. The exon 6B is an alternative exon and maybe this is related. This point should be discussed in the text (e.g. in the section “limitation of the test”).

Response: We added the following to the results:

“The wildtype exons for three exons that undergo alternative splicing in the heart (exons 6B, 18, and 24) had low PSI in the ParSE-seq assay (Figure 2E).”

We added the following to the limitations section of the discussion:

“Exons that undergo extensive alternative splicing in the endogenous tissue (e.g., *SCN5A* exons 6B, 18, and 24) may have low intrinsic PSI in the minigene assay, which may limit the use of the assay for these exons.”

Comment 3: -ACMG Assay calibration: this part is very interesting and rarely present in publication. However, I think that the performances of the test could be overestimated due to the control variants selections. Indeed, most of the P/LP variants selected affected the AG/GT invariants dinucleotide. It is known that Minigene assay is particularly sensitive for this class of variant. However, functional assay was used primarily for variants affecting other positions of splice site than AG/GT. A discussion about the performance of the test should be added in the text.

Response: We agree that assay calibration is a critical, but under used tool. Unfortunately, we are limited in the number of known P/LP splice-altering variants outside of the 2-bp canonical splice site (only 1 P/LP non-canonical splice-altering variant was present in ClinVar). We hope that additional methods development will help better annotate these variants across additional genomic locations. We acknowledge this as a limitation in the discussion as follows:

“Most known P/LP variants for calibration came from canonical splice sites, due to a lack of previously annotated *SCN5A* P/LP splice-altering variants.”

Comment 4: A supplemental table indicating the REVEL and structural penetrance results should be added.

Response: As discussed in our response to Comment 7 below, the REVEL and structural penetrance analysis was attempting to highlight the discordance of missense variant effect predictors focused on protein function versus splicing impact. We were trying to suggest that predictors might incorrectly annotate missense variants, if those variants were to act through an aberrant splicing mechanism. Because this is a difficult to explain and rather tangential point, we have now omitted it from the manuscript to improve readability.

Comment 6: The authors studded 2 missense variants using cell expression experiment (p.T1131I and p.A1407G). How these variants were selected? Both variants had no impact on the protein function but led to splice abnormality. It seemed to be interesting to compare these results with the specific missense/splice in silico predictions.

Response: We provide new electrophysiology data and CRISPR-edited iPSC-CM experiments focused on the c.4220C>G/p.A1407G variant. The updated results section provides an improved flow to increase readability and motivation for experiments as follows:

"In iPSC-CMs, we recovered assignable data for 48 missense variants; 28 were listed as VUS in ClinVar, and 20 were identified prospectively with high SpliceAI scores (>0.8). Of these 48 variants, the ParSE-seq assay identified 18 splice-altering missense variants, created by 20 unique splice-altering single nucleotide variants. These variants were distributed throughout the protein, but often clustered in hotspots near exon boundaries. Most of the splice-altering missense variants were from the prospective high SpliceAI set; only two were found in ClinVar: These variants were both observed in patients undergoing genetic testing in ClinVar and disrupted splicing in the ParSE-seq assay (Figure 6C). We further studied them both by automated patch clamping using HEK293 cells stably expressing *SCN5A* cDNA."

We also now include a more comprehensive analysis of multiple in silico splicing predictors compared to our ParSE-seq results. Please see our response to Reviewer 1, Comment 8.

Comment 7: The comparison of missense impact predictors and ParSe-Seq was questionable. These different approaches were created to detect different effects.

Response: This analysis was attempting to make a minor and rather tangential point and we have now omitted it from the manuscript.

Comment 8: In the text, the authors seemed suggested that ParSe-Seq could be a best approach to cDNA-based assay for missense variant interpretation. Missense variants could impact the protein function, the splicing or both. ParSe-Seq and cDNA-based assay could be complementary for missense variant and maybe in silico tools could guide the choice of functional test ? This point should be discussed in the text.

Response: We agree that a comprehensive approach is best that combines ParSE-seq (to investigate splicing effects) and other functional assays (to assess effects on protein function). Indeed, for missense variants, we did not use a normal splicing result in the ParSE-seq assay to reclassify variants, because we were unable to rule out effects on protein function. We agree that complementary assays should be used to fully annotate variants.

We have updated the results to state:

"Thus, ParSE-seq can help identify a class of missense, splice-altering variants for which cDNA-based assays of protein function yield incorrect conclusions about variant pathogenicity. This result highlights that for missense variants, ParSE-seq can be used to complement traditional cDNA-based assays of protein function."

We now further clarify this point in the discussion:

"We note that for missense variants, we did not use a normal splicing result in the ParSE-assay to reclassify variants, because ParSE-seq cannot rule out effects of missense variants on protein function. We envision that future high-throughput studies of missense variants will use complementary cDNA-based functional assays and ParSE-seq to comprehensively annotate variant effects. *In silico* tools could also be used to prioritize which candidate splice-disrupting variants should be investigated. Alternatively, we demonstrate, albeit in low-throughput for only

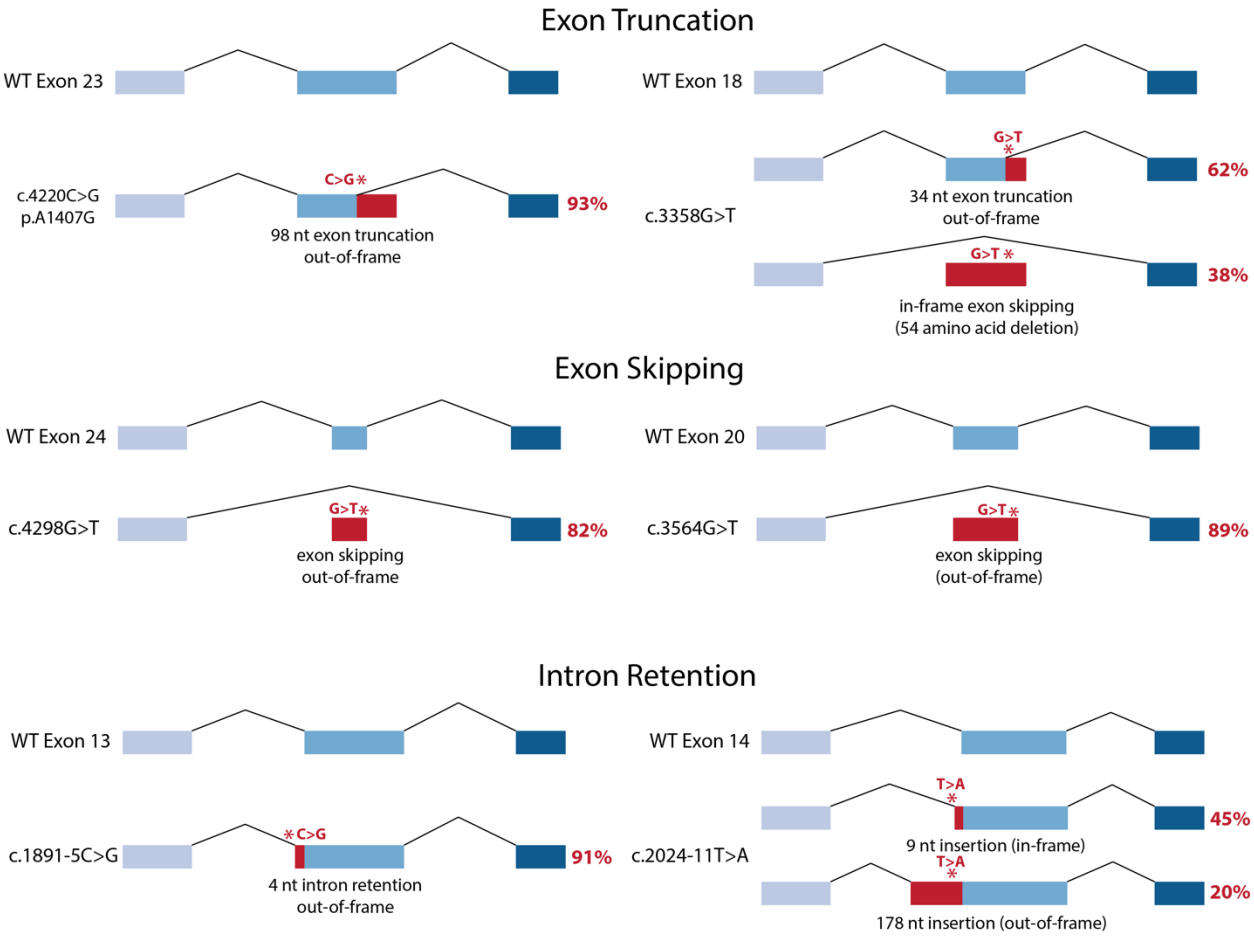
two variants, that variants can be introduced at the endogenous locus by CRISPR into relevant differentiated cell types. These cells can be studied to measure both splice-altering and protein-altering effects (Figure 6)."

Comment 9: No indication about the type of splicing impact (e.g. exon skipping, activation of cryptic splice site) was given in the result section. This point could be important for clinical interpretation notably if the variant led to inframe impact. In this study, the authors seemed to be considered that all splicing impact was necessarily pathological (regardless the inframe or out of frame impact). This point should be indicated in text and argued (maybe no inframe insertion in SCN5A is known as benign)

Response:

Re: splicing impact

We originally focused on ΔPSI_norm to provide a binary splicing outcome for clinical classification purposes in a computationally tractable manner. By annotating reads from the ParSE-seq assay, we are now able to show that non-canonical reads comprise a mixture of intron retention events, exon truncation events, exon skipping events, and (rarely) sequencing errors, consistent with our prior low-throughput study (PMID: 36197721). We now include Supplemental Figure X to highlight examples of various molecular splicing aberrations observed in the ParSE-seq assay:



Supplemental Figure X. Molecular impact of splice-altering variants. ParSE-seq captures exon truncation, exon skipping, and intron retention events. Multiple aberrant splicing events were occasionally observed for a single variant.

We have updated the results:

“To analyze the molecular impacts of splice-altering variants, we examined the composition of non-canonical splicing reads (Supplemental Figure X). We observed exon truncation events, intron retention events, and exon skipping events, consistent with our prior low-throughput study (PMID: 36197721). For some variants, multiple aberrant splicing events were observed at appreciable levels. Many of these altered transcripts resulted in changes to the reading frame. In addition, some small and large in-frame insertions and deletions, were also observed.”

Consistent with the Response to Reviewer 3, we have updated the limitations to state:

“While ParSE-seq quantifies broad molecular impacts such as exon skipping, exon truncation, and intron retention, it is possible that some splicing abnormalities may not have a detrimental effect on downstream protein function (protein tolerant in-frame insertion/deletions). In the current version of gnomAD v4, there are no observed indel variants >3 amino acids that are reported as benign/likely benign.”

Comment 10: Recapitulating Splice Effect at the endogenous locus: as the splicing impact was confirmed using CRISPR for only one variant, this part did not seem to be essential. If the authors want to keep it, the choice of the variant should be argued. Moreover, in the discussion section, the place of CRISPR assay comparing to in silico/ ParSe-Seq should be discussed.

Response: We used CRIPSR edited iPSC-CMs to help validate the ParSE-seq results. These experiments are laborious and expensive so we were limited in how extensively we could pursue these experiments. As the reviewer notes, our initial submission included only a single intronic variant that was studied in CRISPR edited iPSCs. This variant was a randomly selected intronic variant reported in a heterozygote undergoing genetic evaluation for Brugada Syndrome.

We now present a second variant, p.A1407G, that has been modeled in CRISPR edited iPSC-CMs at the endogenous locus. This variant was chosen because it was a missense variant that appeared in a patient but we show that it acts through a splicing mechanism. Please see our response to Reviewer 3, Comments 4 and 9 for a full description of this experiment. We have updated the main figures to include the cDNA assay and iPSC-CM assay for p.A1407G. We have moved the previous intronic CRISPR variant to Supplemental Figure XV.

We have added the following to the results:

“The c.4220C>G/p.A1407G variant was chosen for functional assessment in CRISPR-edited iPSC-CMs to further validate the finding of a missense variant acting through a splicing mechanism.”

We have added the following to the results:

“To further validate the ParSE-seq assay, we studied an intronic variant by CRISPR-editing of control iPSC-CMs (Supplemental Figure XV). This variant was selected from the set of splice-

disrupting variants that appeared in patients. We found concordance between the ParSE-seq predictions of aberrant splicing and iPSC-CM RNA-splicing, with introduction of a frame-shifting 4-base pair intron retention event. Moreover, this aberrant splicing event severely abrogated peak current density by patch-clamp of the iPSC-CMs, consistent with the primary mechanism of *SCN5A*-BrS (Supplemental Figure XV)."

We have added the following to the discussion:

"We performed validation of the ParSE-seq results in iPSC-CMs for two variants. In both cases, the cardiomyocytes had similar changes to splicing as the ParSE-seq assay and reduced sodium currents. However, comprehensive CRISPR-edited iPSC-CM validation of dozens or hundreds of variants using this method is currently impractical."

Comment 11: -Line 386-401 (Methodology Overview and feasibility) : this paragraph largely overlapped with tee Method section without presenting any results. This paragraph should be removed from the text and the Figure 1 should be introduced throughout the Method section. The same remark could be made for the following paragraph (Assay implementation). The results started to be presented only from line 411.

Response: In response to reviewer 3, we aimed to ensure that adequate detail of the methods would also be presented in the results section. We have added references to Figure 1 and several new supplementary figures throughout the Methods section to increase readability for the general audience. We are happy to defer to the editor for the preferred presentation in *Nature Communications*.

Comment 12: In supplementary Table IV and V, the column "ClinVar Clinical Significance" should be added for easier reading

Response: We updated Supplemental Tables IV and V as suggested (current Tables V and VI).

Comment 13: The figure 2A largely overlapped with the Figure SI. These two figures should be merged

Response: We removed the SI figure and renumbered the figures accordingly.

Reviewer #3

O'Neill et al study splicing effects of mutations in the arrhythmia-associated SCN5A gene using a multiplexed minigene reporter assay. They relate the measured splicing effects to own electrophysiological measurements and to clinical evidence relating mutations to disease outcome in Brugada syndrome.

To systematically test for splicing changes within disease-associated genes, the authors introduce ParSE-seq, by which they study how the splicing of almost 20 exons is affected by exonic and nearby intronic mutations. For the SCN5A gene, they report a strong correlation of measured splicing effects with in silico predictions and with known clinical effects of the variants. In a more exploratory part, they use their method to classify variants of unknown clinical significance and functionally test three selected mutants.

While the initial screening results are interesting and convincing, the relevance of the validation part remains much less clear (see below). Furthermore, the study remains mostly descriptive with little focus on molecular mechanisms. Finally, the presentation of the results should be improved, as the rationale and description often remain vague.

Comment 1: The reporter constructs were built based on separated individual exons of SCN5A, embedded in an exogenous sequence context. Therefore, splicing outcomes in the minigene reporter likely deviate strongly from alternative splicing patterns of the endogenous SCN5A pre-mRNA. Some potentially relevant splicing outcomes (like intron retention) likely cannot be analyzed at all in a meaningful way.

Response: We have now performed an additional analysis quantifying each class of aberrant splicing detectable in our assay, including intron retention, exon skipping, and exon truncation. Please see our response to Comment 4 below for a full description of this analysis.

Comment 2: The authors should clarify how their results link to normal splicing behavior of SCN5A exons? Is the PSI of the reporter constructs similar to the PSI of the endogenous exons? Why is no exon included more than 75% (Fig. 2E)?

Response: Although we include up to 250 bp on each side of intronic sequence, minigene reporter constructs may still have fewer regulatory motifs than endogenous genomic loci. Each insert harbors different linear combinations of *cis*-regulatory Exonic Splicing Enhancer/Exonic Splicing Silencer and Intronic Splicing Enhancer/Intronic Splicing Silencer motifs. These regulatory motifs will influence the strength of each exon's recognition by each cell type's *trans*-acting spliceosome, resulting in different WT exon PSIs.

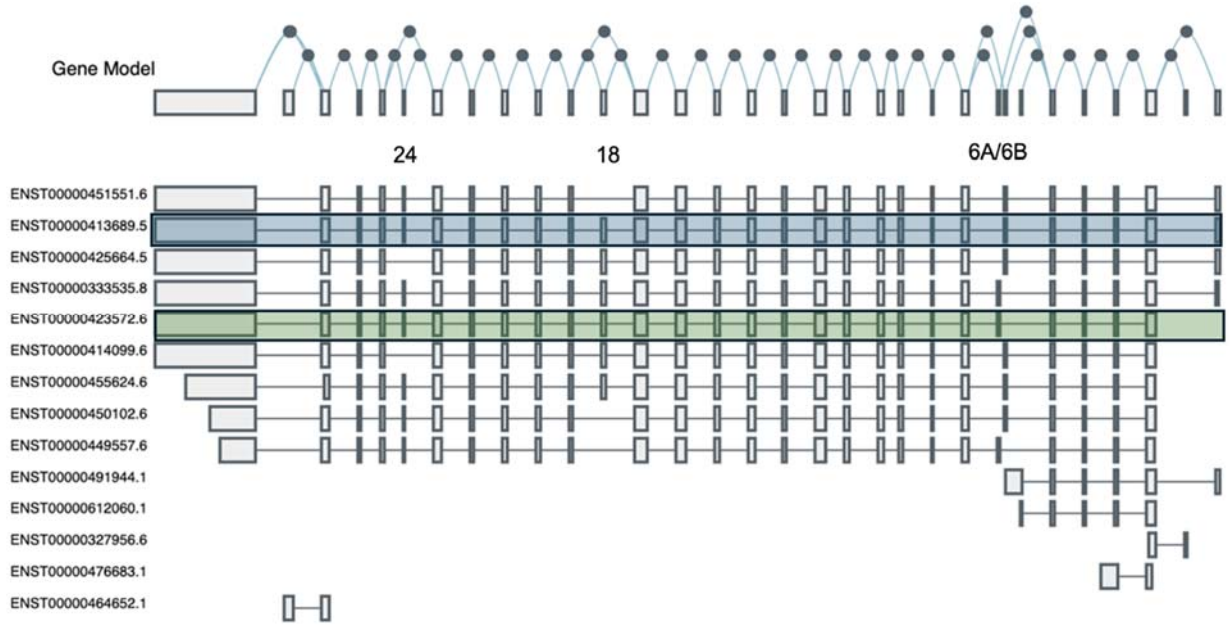
SCN5A is a gene that undergoes extensive alternative splicing with multiple primary transcripts in the human heart and other tissues (see new Supplemental Figure VIII). The exons with the lowest WT PSI in the ParSE-seq assay are the same exons that undergo extensive alternative splicing in humans (exons 6B/18/24). For example, exon 6B alternative splicing is tightly regulated by tissue-specific *trans*-acting proteins that change throughout aging (PMID: 27063795), which would not be completely recapitulated by a minigene assay.

Despite including longer intronic sequences to include additional *cis*-regulatory motifs, we did not observe exons that had baseline PSI >75%. As non-alternatively spliced exons are constitutively spliced in across the most clinically relevant *SCN5A* transcripts (~100% PSI; PMID: 35388217), these results are consistent with incomplete capture of all *cis*- and *trans*-

regulatory elements within a native human cardiomyocyte. However, our calibration on known controls supports the robustness of the assay for clinically-relevant variants.

We have updated the limitations to state:

“Exons (e.g., *SCN5A* exons 6B, 18, and 24) that undergo extensive alternative splicing in the endogenous tissue may have low intrinsic PSI in the minigene assay, which may limit the use of the assay for certain exons.”



Supplemental Figure VIII. *SCN5A* transcript alternative splicing patterns from the Genotype Tissue Expression Portal (GTEx; PMID: 29022597). *SCN5A* is transcribed right to left, across exons 1-28. Clinically relevant transcripts are ENST00000423572.6 (Green; MANE select) and ENST00000413689.6 (Blue; MANE clinical plus). The alternative spliced exons 6A/6B, 18, and 24 are depicted above the transcript tracks.

Comment 3: Why Rat IR exons were chosen as flanking sequences? How do these sequences, e.g., possible ESE/ESS sites within them, affect splicing behavior compared to the endogenous gene?

Response: We chose to use the pET01 splicing vector because this is a commonly used minigene plasmid that we (PMID: 36197721) and others use. To our knowledge, ESE/ESE and ISE/ISS sites in this plasmid have not been investigated. To mitigate effects of the rat ESE/ESS sites on splicing of our exons, we tried when possible to include up to 250 bp of intronic sequences from the endogenous locus on either side of the test exon. We hoped that this would capture relevant splicing enhancer sequences near the test exon, and minimize the effect from rat IR intronic/exonic sequence which was typically >250 bp from the test exon. This large endogenous intron size in our experiment is a positive feature of our approach in contrast to previous methods that used only very small intronic segments from the endogenous locus.

We have edited the limitations to read:

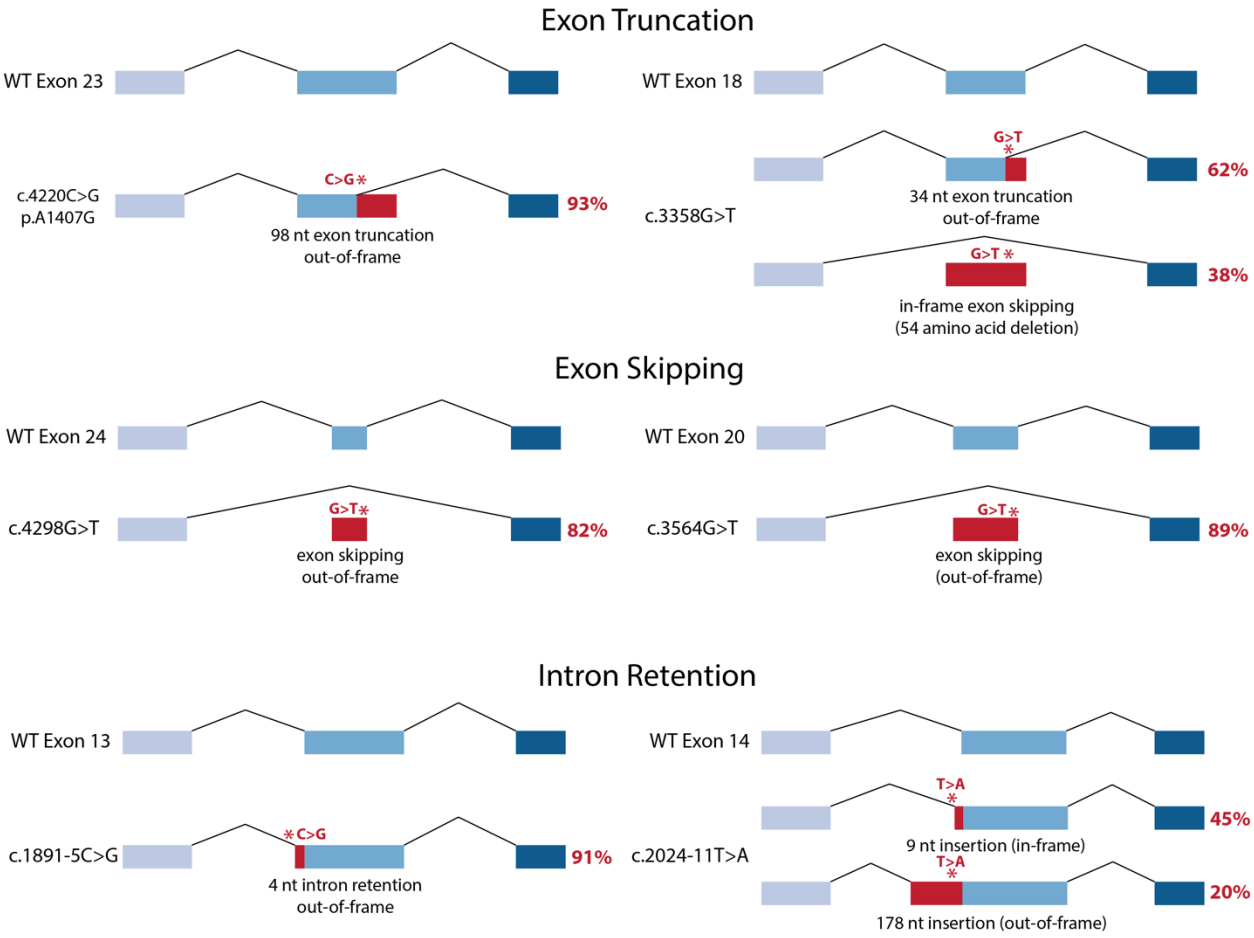
“There may be examples where the ParSE-seq minigene assay does not fully capture all nuances of biology at the endogenous locus. For example, splicing regulatory motifs in the native context may have a long-distance effect not captured in the minigene-based assay.”

And

“Although we validate two ParSE-seq splice-altering variants by CRISPR editing of the iPSC-CMs, most variants were tested only in multiplexed minigene assays.”

Comment 4: The authors should more clearly state which splicing fates are covered by their minigene and which ones cannot be studied (likely the case for intron retention, skipping of multiple exons at a time, or mutually exclusive exon inclusion which frequently contribute to pathological outcomes as well).

Response: We originally focused on ΔPSI_norm to provide a binary splicing outcome for clinical classification purposes in a computationally tractable manner. By annotating reads from the ParSE-seq assay, we are now able to show that non-canonical reads comprise a mixture of intron retention events, exon truncation events, exon skipping events, and (rarely) sequencing errors, consistent with our prior low-throughput study (PMID: 36197721). We now include Supplemental Figure X to highlight examples of various molecular splicing aberrations observed in the ParSE-seq assay:



Supplemental Figure X. Molecular impact of splice-altering variants. ParSE-seq captures exon truncation, exon skipping, and intron retention events. Multiple aberrant splicing events were occasionally observed for a single variant.

We have updated the results:

“To analyze the molecular impacts of splice-altering variants, we examined the composition of non-canonical splicing reads (Supplemental Figure X). We observed exon truncation events, intron retention events, and exon skipping events, consistent with our prior low-throughput study (PMID: 36197721). For some variants, multiple aberrant splicing events were observed at appreciable levels. Many of these altered transcripts resulted in changes to the reading frame. In addition, some small and large in-frame insertions and deletions, were also observed.”

Currently, we are studying single insert constructs, so the ParSE-seq assay cannot detect multiple exon skipping or mutually exclusive exon inclusion events.

We have updated the discussion to say:

"Most minigene-based methods are limited by which constructs they can study. Mutually exclusive exon splicing and multiple exon skipping events are increasingly recognized as causes of disease (PMID: 36747048, 38658687). Although our current study focuses on a single study exon and surrounding intronic sequence, the ParSE-seq method should be amenable to inclusion of multiple study exons, constrained by cost of synthesis and transfection efficiency of large plasmids.”

Please see our response to Reviewer 2, comment 9 about the consequences of in-frame insertions/deletions.

Comment 5: From just reading the main text and figures, many aspects of the minigene assay and results are not clear. The authors should briefly explain the following aspects in the main text without the necessity that readers need to check this in the Supplement/Methods or previous work.
- Which regions/exons in SCN5A were studied? Why was only a subset of exons studied in the end?

Response: We revised the methods for clarity to state:

“The *SCN5A* transcript ENST00000333535.9 contains 27 coding exons (exons #2-28). We interrogated splicing effects of variants in 19 of these exons (Figures 3B and 3C). The minigene-based assay requires an acceptor and donor splice site on each end of the test exon, and is therefore incompatible with the first or last coding exons (2 and 28). In addition, *SCN5A* uses 2 instances of non-canonical AC/AT splice sites between exons 3 and 4, and exons 25 and 26. Therefore, we did not study variants in these 4 exons or in adjacent intronic locations. Furthermore, we were unable to include plasmids with exon 15 due to synthesis incompatibility (high GC content) and exon 17 due to overlap of restriction enzymes used for barcoding.”

We have also added the following to the results: “We studied splicing effects of variants in 19 of the 27 coding exons of *SCN5A*. These exons were chosen based on their compatibility with the minigene assay (use of canonical splice sites and flanking exons), Twist Clonal Gene synthesis, and restriction digestion compatibility.”

We added a sentence to the limitations: “The first and last exons of a gene, exons using non-canonical 2-bp splice sites, and exons that were difficult to synthesize due to high GC content or restriction enzyme incompatibility were not included in the library.”

We have made several additional changes throughout the text to simplify and improve readability, as described in other Reviewer responses.

- How were mutations chosen and how was the mutagenesis performed?

Response: As was also described in our response to Reviewer 2, comment 1, this information has now been addressed in the results as follows:

“We studied variants from two groups: 1) exonic and intronic variants clinically observed in individuals; and 2) exonic variants predicted by SpliceAI to disrupt splicing (Figure 1F). Clinically observed variants came from gnomAD, ClinVar, and literature reports (some of which were not present in ClinVar). B/LB and P/LP variants from ClinVar were used to calibrate the assay (see methods; N = 69), after which we applied calibrated functional evidence criteria to facilitate classification of VUS and Conflicting Interpretation Variants (N = 119). These included 258 single nucleotide variants and 12 indels. There were 58 exonic single nucleotide variants with high aggregate SpliceAI scores (>0.80), of which 3 had been observed in ClinVar (2 VUS, 1 Conflicting Interpretation).”

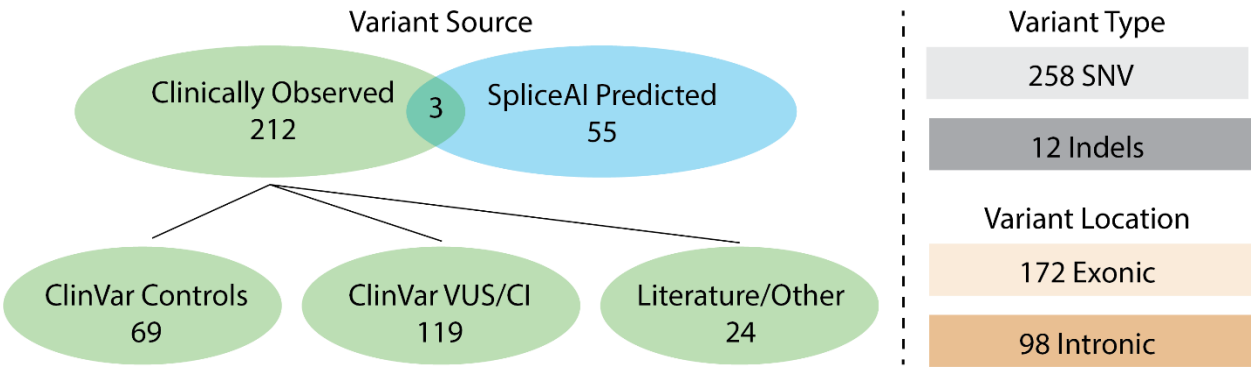
Please see our updated response to Reviewer 1 comment 1 and 3 for newly improve mutagenesis details and schematics of the cloning process.

- The authors should specify in the text the kinds of "variants" they are investigating (e.g. SNPs, Indels, etc.) and show how frequently these variants occur in patients.

Response:

Re Variant Type:

We have updated Figure 1E:



The methods now read:

“Variants included 258 single nucleotide variants and 12 insertions/deletions (indels).”

Re Variant Frequency in Patients:

The frequency/prevalence of variants among patients is difficult to address for rare diseases. Many *SCN5A* variants identified in this study were taken from ClinVar and previous literature reports (see Reviewer 2, Comment 1). Unfortunately, ClinVar does not provide detailed patient counts to assess frequency; moreover, there is a difficult problem of incomplete penetrance among the channelopathies (PMID: 36496179). These difficulties prevent us from comprehensively addressing variant prevalence among affected heterozygotes.

To still address this question with available data, we interrogated the frequency of splice-altering variants in a large cohort study of 614 *SCN5A*-Brugada Syndrome patients harboring a variety of *SCN5A* variants, i.e. missense, splice-site, frameshift, nonsense, intronic (PMID: 32893267).

We have updated the methods to say:

“We included Brugada Syndrome patient case counts when available from a previously published international cohort study (PMID: 32893267). For ClinVar variants, we assumed at least one affected patient per reported variant”.

We have updated the results to state:

“In a recently published international Brugada Syndrome patient cohort (PMID: 32893267), there were 614 *SCN5A*-Brugada Syndrome patients harboring a variety of *SCN5A* variants, i.e. missense, splice-site, frameshift, nonsense, intronic. Of these, there were a total of 27 splice-altering variants affecting 43 patients (functionally abnormal non-consensus splice variants or consensus splice site variants). In the current study, we proactively investigated 18 unique variants harbored by 36 patients, which complemented our previous low-throughput investigations (PMID: 36197721; see updated Supplemental Table III).”

We have updated the limitations to state:

“The frequency of affected variant heterozygotes is difficult to ascertain based off ClinVar data alone, as detailed patient phenotypes and case counts are not routinely reported from submitting centers”.

- Please briefly explain dPSI_norm in the main text. In what sense is this normalized?

Response: We aimed to normalize each variant construct to the WT construct to account for the variability of PSI for each WT construct (Comment 2 above). We have edited the methods to read:

“For each variant, a normalized ΔPSI value was calculated:

$$\Delta PSI\_norm_{variant} = (mean(PSI_{barcode,variant}) – mean(PSI_{barcode,WT}) / mean(PSI_{barcode,WT})$$

This ΔPSI_norm value represents the change in splicing of the variant compared to the level of splicing of the corresponding wildtype exon. The value is normalized to the level of wildtype splicing to determine the percent change of splicing regardless of the baseline level of wildtype

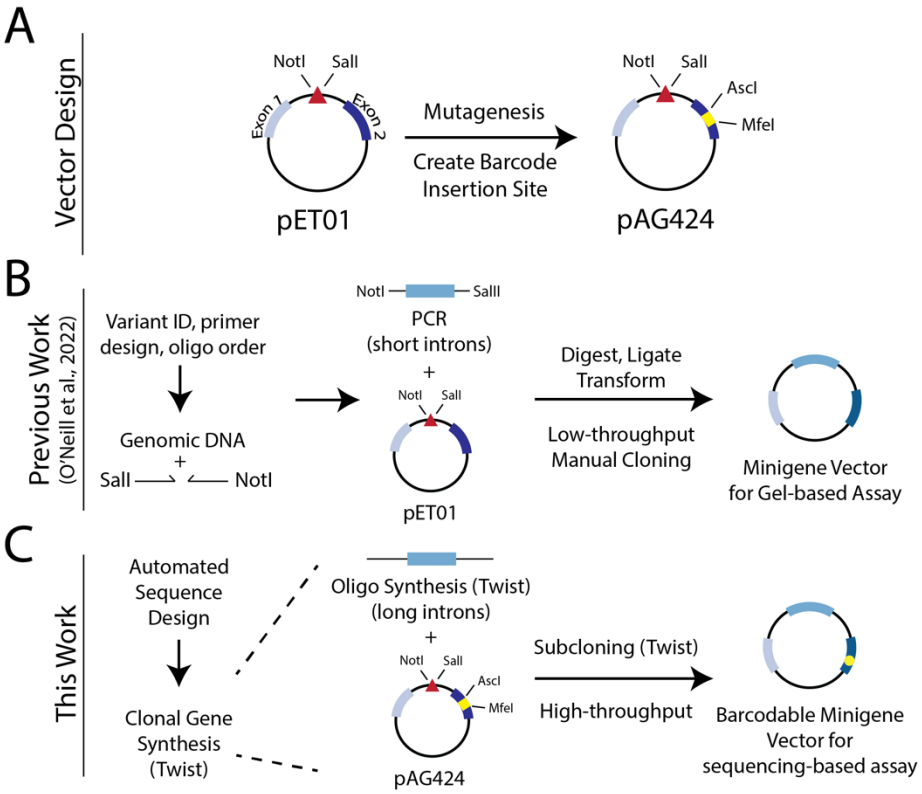
PSI. For example, if a wildtype exon had a PSI of 80% and the variant exon had a PSI of 40%, the ΔPSI_norm would equal -50%."

- Please also briefly explain some aspects of the reporter system in the main text: another plasmid/vector system, pAG424, is mentioned and described to be part of the plasmids used for splicing analysis of SCN5A. However, it is unclear from the methods and/or (supplementary) figures how the two vectors pET01 and pAG424 are related.

Response: We have updated the results to clarify this point and provide further context to the reader.

"Accordingly, we adopted a previously published minigene to incorporate a site for barcoding to enable multiplexed experiments (Figure 2A). Specifically, we used the established minigene vector pET01 (MoBiTec GmbH) and created a restriction site in the downstream exon to allow for insertion of barcodes. We refer to the resulting plasmid as pAG424. Barcoding at this site enables multiplexed, high-throughput sequencing-based quantification of splicing effects, rather than gel-based quantification in our previous study (PMID: 36197721; see Supplemental Figures I and II for complete schematics). In this work, we first used long-read sequencing of the barcoded plasmids to associate barcodes to wildtype or variant-containing plasmids. We then used short-read target RNA-seq to quantify splicing patterns for each plasmid (PSI; Figure 2B)."

We also provide a new Supplemental Figure I which schematically depicts this process.



Supplemental Figure I. Schematic of vector design and cloning overview.

A) pET01 is an established minigene vector containing a MCS between rat insulin exon 1 and 2 and flanking intronic sequences (MoBiTec GmbH). We used PCR-mutagenesis to create a new restriction site in rat insulin exon 2 to allow insertion of a barcode.
B) Schematic of previously described workflow for manual minigene assays using gel-based quantification and manual cloning (PMID: 36197721).
C) Schematic of current approach for a sequencing-based quantification of splicing. pAG424 was sent to Twist Biosciences, and non-restriction based cloning of chemically synthesized oligonucleotides was performed.

Comment 6: The dPSI is known to depend on the starting PSI (Baeza-Centurion et al., 2019; Braun et al., 2018).
- Is this finding true in the present dataset as well? (How) do the conclusions change if the starting PSI is taken into account?

Response: The WT *SCN5A* exon cassettes had variable PSI at baseline in both cell types, HEK and iPSC-CM (Figure 2C). This sequence context variability is consistent with the results in the recommended references (PMID: 30661752 and 30120239), as each construct will have different linear combinations of *cis*-regulatory Exonic Splicing Enhancer/Exonic Splicing Silencer and Intronic Splicing Enhancer/Intronic Splicing Silencer motifs. If we had used an absolute change in PSI, then the results would be biased against exons that have weaker baseline PSI. To overcome this challenge and ensure consistency of conclusions across variable PSIs, we used a normalized change in PSI to account for variability (see the next comment for further details). We also note that many exons in the endogenous environment of the heart are likely partially mis-spliced at baseline, but these aberrant splice forms are eliminated by Nonsense Mediated Decay. We observe this phenomenon in our iPSC-CMs by RNA-seq. Wildtype cells treated with the NMD inhibitor cycloheximide have a small degree of mis-splicing for many exons, and minimal mis-splicing without the NMD inhibitor treatment.

- Why do the authors dPSI_norm > -20% assumed to be ‘normal’? In the methods part, the dPSI_norm formula probably lacks a factor of 100.

Response: We now provide a sensitivity analysis using alternate cutoffs in our response to Reviewer 1, Comment 7. This analysis demonstrates that using alternate cutoffs did not change the major conclusions of the study.

As recommended, in order to make the values percentages, we edited the PSI formulas to include a factor of 100 during the initial calculation of PSI:

$$\text{“}PSI_{barcode} = 100 \times \#\text{normal reads}_{barcode} / \#\text{total reads}_{barccode}$$

computationally implemented as:

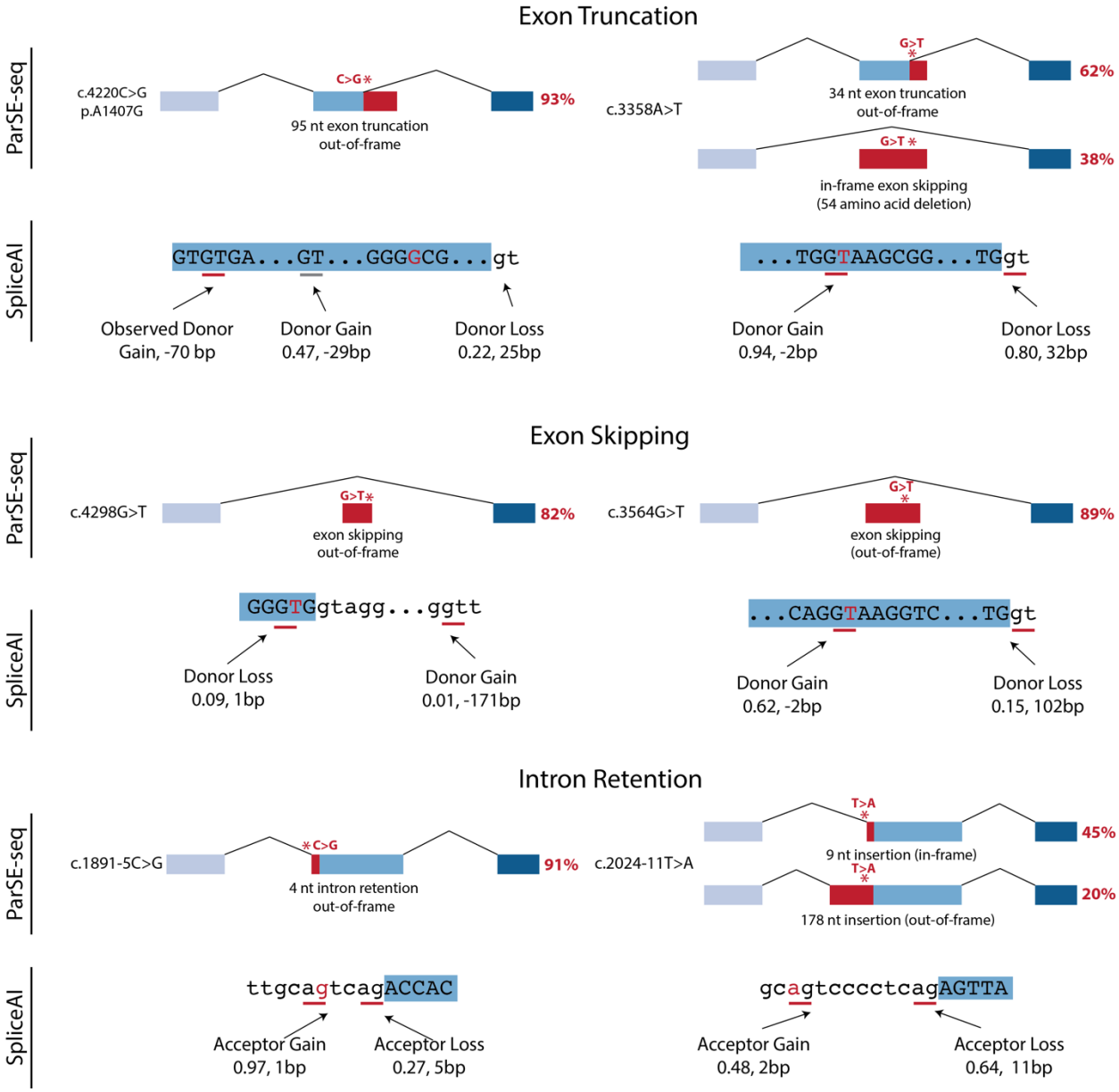
$$PSI_{barcode} = 100 \times \min(PSI_{barcode,\ R1}, PSI_{barcode,\ R2})/\text{total reads}_{barcode}\text{”}$$

This adjustment results in the downstream values ΔPSI and $\Delta PSI\_norm_{variant}$ also being increased by a factor of 100 (converted to percentages).

Comment 7: Functional study and SpliceAI: The authors find an impressive concordance of SpliceAI predictions and their experimental findings.
- To allow the readers to better judge these results, they should describe in more detail what the

(aggregated) SpliceAI scores actually predict in terms of molecular splicing events and what actually happens at the molecular (junction) level in their assays (beyond changes in the PSI metric that combines many different molecular changes).

Response: To provide examples of the SpliceAI predictions versus experimentally derived outcomes, we added a new Supplemental Figure XIII:



Supplemental Figure XIII. SpliceAI predictions and ParSE-seq molecular outcomes. We compared ParSE-seq experimental outcomes (top) with SpliceAI predictions for donor/acceptor loss/gain (bottom) across three different molecular events. SpliceAI gives one prediction for each variant, where some variants led to both the SpliceAI prediction and exon skipping. SpliceAI predictions typically matched experimental data for activation of cryptic splice sites (c.3358A>T, c.1891-5C>G, c.2024-11T>A), although in the ParSE-seq assay a different cryptic

site may be activated (c.4220C>G) or exon skipping may predominate (c.4298G>T and c.3564G>T).

We have updated the results:

“In addition to aggregate SpliceAI scores and ParSE-seq ΔPSI_norm comparisons across the library, we also compared specific SpliceAI molecular predictions to observed ParSE-seq splice outcomes (Supplemental Figure XIII). SpliceAI predictions typically matched experimental data for activation of cryptic splice sites (c.3358A>T, c.1891-5C>G, c.2024-11T>A), although in the ParSE-seq assay a different cryptic site may become activated (c.4220C>G) or exon skipping may result (c.4298G>T and c.3564G>T). Some variants led to multiple splice aberrations in the ParSE-seq experiment (e.g. exon skipping and exon truncation/intron retention), despite only having a single SpliceAI predicted aberrant event (c.3358A>T and c.2024-11T>A).”

We have updated the discussion to state:

“Our current implementation of splice prediction tools aggregates predictions of acceptor and donor loss/gain to a single value to enable comparisons across tools. However, manual interrogation of such predictors may provide insight into exact molecular vs *in silico* predictions of spliced RNA (Supplemental Figure XIII). A recently published tool allows for the visualization of any SpliceAI prediction to facilitate molecular interpretation (PMID: 36765386).”

- Since the authors want to focus also on splice variants with a disruption that is not at the canonical 2-bp splice-site motifs, wouldn’t it be more reasonable to show Suppl. Fig VIII in the main figures instead of Fig4B? While in Fig4B R^2 is higher than in SFigVIII, the majority of data points seem to accumulate at the extremes, which not only makes Fig4B look more noisy, but also looking at SFigVIII, these data points are mostly disruptions of the canonical splice sites, which is somewhat trivial.

Response: We agree. We have moved former Supplemental Figure 8 to the main text and updated the corresponding figure legend:

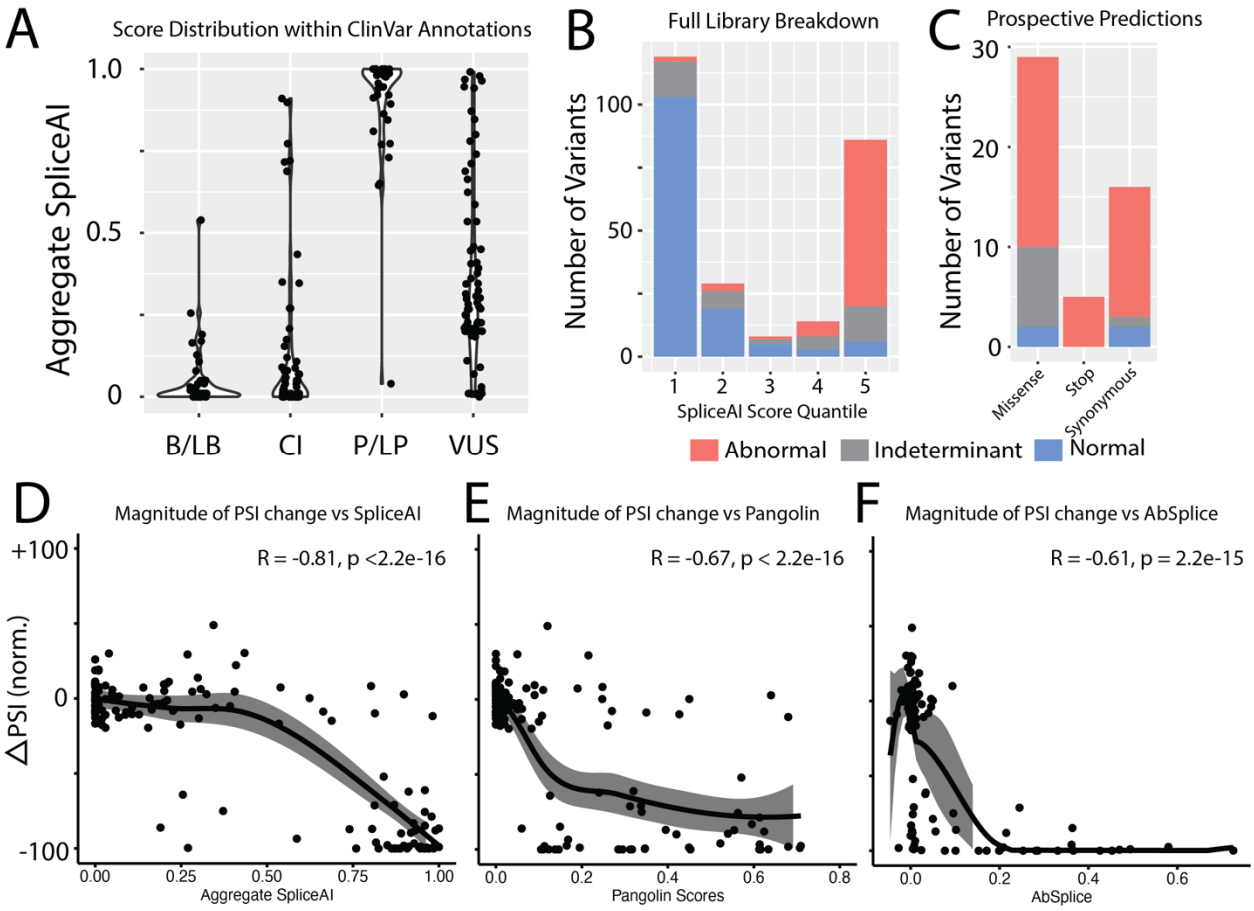


Figure 4. Comparison of experimental data and *in silico* splicing predictors.
A) Aggregate SpliceAI scores for each ClinVar variant class.
B) Distribution of variant effect in ParSE-seq stratified by SpliceAI score quintiles.
C) Results of prospectively identified exonic variants by SpliceAI score >0.8 stratified by mutation type and ParSE-seq outcome.
D-F) Correlation of normalized ΔPSI for non-canonical splice site variants against aggregate SpliceAI scores (D), Pangolin scores (E), and ABSplice scores (F). Confidence interval fit using LOESS (see methods).

- How do the authors comment on the outliers in Fig4B and SfigVIII (lower left and upper right points)? The discrepancy is mainly due to limitations in SpliceAI algorithm or ParSE-seq assay?

Response: We are unable to distinguish whether discordant variants are primarily due to the limitations of the experimental or *in silico* predictions without additional external validation. A previous benchmarking of *in silico* splicing prediction tools show that SpliceAI and others are still imperfect, especially for exonic variants (PMID: 38129864); however, ParSE-seq also has its own experimental limitations (for example being a minigene assay). Future work could further investigate these discordant variants, for example in an iPSC-CM model or in patient cohorts.

We have updated the limitations as follows:

"There may be examples where the ParSE-seq minigene assay does not fully capture nuances splicing regulation at the endogenous locus. This incomplete ascertainment may lead to discordant results with *in silico* predictors for a subset of variants."

- The details of LOESS fitting, e.g., brief method introduction, key parameters, etc., needs to be added in the method section.

Response: We have added the following to the methods:

"We used locally estimated scatterplot smoothing (LOESS) as a non-parametric regression model for comparing *in silico* splicing predictors with experimental ParSE-seq data. LOESS was selected as a smoothing method due to the largely bimodal distribution of our experimental data. A 95% confidence interval is displayed alongside the line of fit. LOESS was implemented in ggplot2 using default settings with $\Delta PSI\_norm_{variant}$ plotted as a function of aggregate SpliceAI predictions. Full code describing this analysis is available on GitHub."

Comment 8: In the sections "ACMG assay calibration" and "variant reclassification", the authors seem to compare their splicing results to previously reported variant association with clinical phenotypes.
- While the comparison to variants of known clinical significance seems impressive, the authors should explain in more detail the used ClinVar classification. Is it really expected that each splicing defect has a strong impact on protein function? To justify this assumption that also underlies the subsequent variant reclassification, the authors should provide more molecular details about splicing alterations in the studied variants and should discuss their expected effect on protein function and expression level.

Response: A major goal of the current study was to provide new experiment data ("functional data") to directly facilitate the classification of variants within ClinVar. In a 'genotype-first approach', we supplied these experimental data irrespective of clinical phenotypes. We have striven to follow the most stringent interpretation of ACMG functional assay guidelines. The ClinGen Sequence Variant Interpretation working group recommended calibrating functional assay evidence strength based on assay performance on large numbers of B/LB and P/LP control variants (PMID: 31892348). Therefore, we aimed to study 69 B/LB and P/LP variants obtained from ClinVar. After calibrating the assay, we then applied the appropriate evidence strengths to VUS and Conflicting Interpretation Variants.

We now provide an expanded discussion and additional data exploring the molecular impact of various aberrant splicing outcomes and their impact on protein function. Please see our response to Reviewer 3 Comment 4 and Reviewer 2 Comment 9 above.

Comment 9: - How robust are the reclassification results? Can the prediction power be tested computationally, e.g., cross validation, or by other experimental/clinical data?

Response: The ParSE-seq results on 47 and 58 control benign and pathogenic variants in iPSC-CMs and HEK cells, respectively, provide functional strong evidence supporting variant reclassification, as recommended by the ClinGen Sequence Variant Interpretation working group (PMID: 31892348). This approach examines the performance of the assay on control variants to generate an Odds of Pathogenicity. The Odds of Pathogenicity guides the strength of evidence, in this case functional, that is then applied to the non-control variants. The ParSE-seq

assay showed excellent performance on the control variants, which when quantified led to us applying the PS3 and BS3 criteria at a strong level to classify variants.

To experimentally validate our ParSE-seq findings, we also performed two orthogonal CRIPSR iPSC-cardiomyocyte experiments studying variants at their endogenous locus. Both of these CRISPR lines were highly concordant with the splicing disruptions predicted by ParSE-seq. We plan to perform more of these experiments in the future, although they are time- and resource-intensive.

We have updated the discussion:

“There is only limited clinical data currently available for most of the variants in this study, and some of the variants have not yet been detected in any individuals to our knowledge (high SpliceAI prediction variants). However, with the rapid rise of genetic sequencing and data sharing (especially regarding patient phenotypes), many future individuals will likely be discovered with variants in this study. These phenotypes of these individuals could be examined to further examine the validity of our variant classifications.”

Comment 11: The rationale underlying Fig. 6 should be explained in more detail:
What kind of splicing effects do the authors specifically think of that are not captured by the cDNA assay? The authors perform patch-clamp assays and show that the two studied variants have normal electrophysiological function, Then, they employ in silico analyses (of unclear relevance) to conclude that these are “splice-altering variants for which cDNA-based assays of protein function yield incorrect conclusions”. In its present form, this part of the paper is not convincing.

Response: In this section, we make the argument that traditional patch clamp assays using cDNA do not capture splicing effects. cDNA is composed of already spliced-together exons. Any intronic or exonic variant causing exon skipping, intron retention, or exon truncation in its native context will not be detected.

To further strengthen our argument, we now provide new data for the missense variant p.A1407G. This variant disrupts splicing in the ParSE-seq experiment but has near-normal function in a patch clamp assay in HEK293 cells using cDNA. Thus, we hypothesized that this variant acts through a splicing mechanism, as opposed to altering protein function due to the amino acid change. In this revision, we now present new data showing a CRIPSR introduced iPSC-cardiomyocyte model of the missense variant p.A1407G at the endogenous locus. By RNA-seq, this variant line had highly altered splicing of *SCN5A*, matching the observation from the ParSE-seq data. In addition, the line had reduced sodium currents as measured by patch clamping. This variant is now a fleshed-out example of a missense variant that disrupts protein function through aberrant splicing.

The new data is presented in an updated Figure 6 (below):

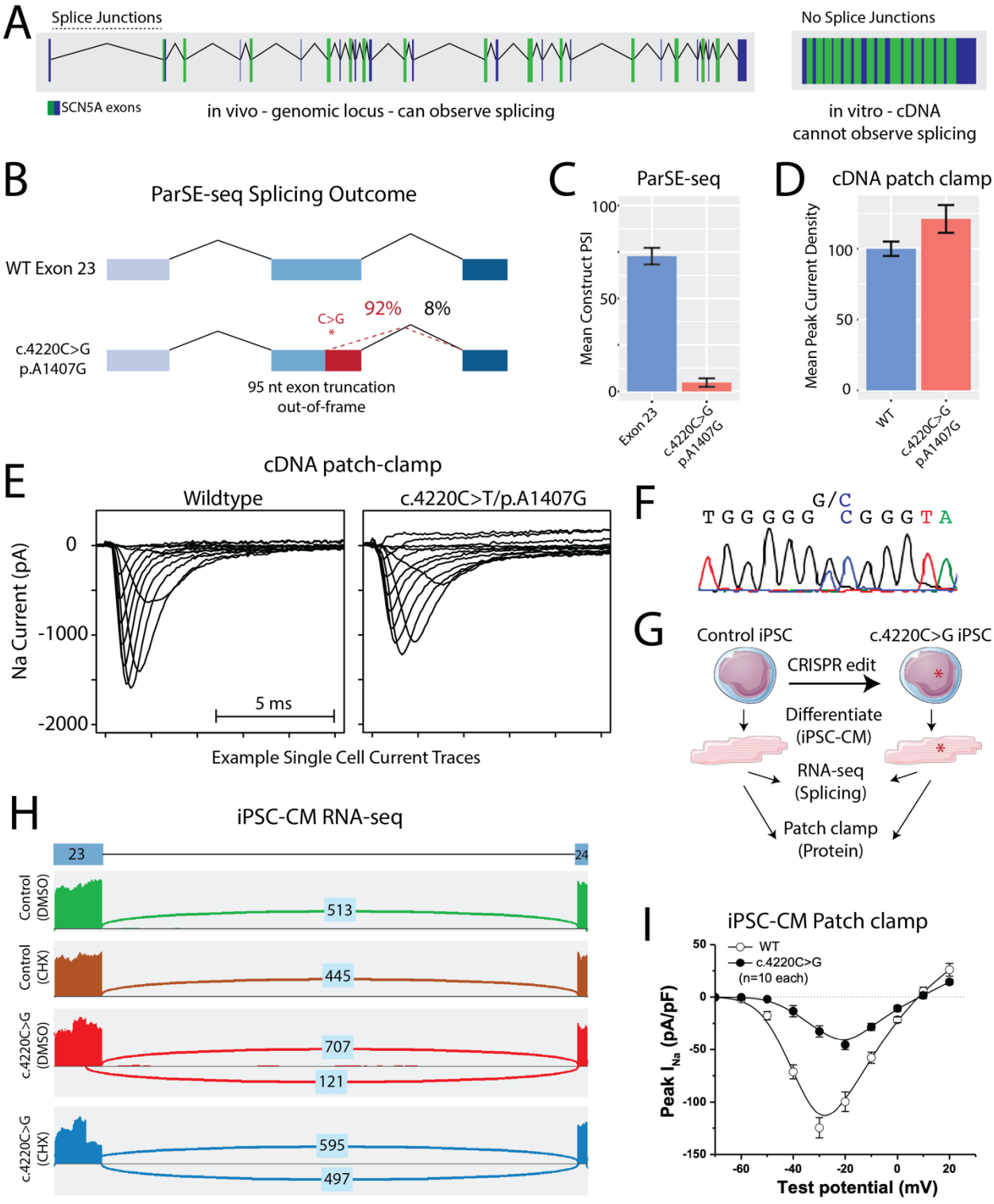


Figure 6. Multi-assay investigation of an *in vitro* splice-altering missense variant.
A) Schematic showing that cDNA-based assays do not account for splice-altering variant effects. Left: Schematic of genomic locus with large intronic sequences; Right: cDNA-based sequence without introns used in many *SCN5A* functional assays. Alternating exons are annotated in green and blue.

B) Molecular analysis of the ParSE-seq assay showed activation of an upstream cryptic splice donor site, resulting in a 31-bp exon truncation.
C) Quantification of mean canonical PSI among reads for the WT exon construct and variant construct. Error bar corresponds to standard error of the mean.
D) Quantification of sodium channel current densities for WT $Na_V1.5$ and variant $Na_V1.5$ using the SyncroPatch automated patch-clamping system (cDNA assay), in stably expressing HEK293 cells. Error bar corresponds to standard error of the mean.
E) Representative single cell sodium current traces for a WT and variant HEK cell. cDNA assessment of this missense variant did not show an effect on protein function when assessed by automated patch-clamping, a system that cannot assess splicing impact.
F) CRISPR editing of a population control induced pluripotent stem cell (iPSC) line was performed to make a heterozygous edit of the line.
G) The WT and heterozygote variant iPSCs were chemically differentiated into cardiomyocytes (iPSC-CMs).
H) Differentiated iPSC-CMs were treated with DMSO or the NMD inhibitor cycloheximide, followed by RNA-isolation and RNA-seq. We observed aberrant splicing consistent with the ParSE-seq molecular event (exon truncation) in the variant, but not WT lines. Notably, treatment with cycloheximide increases the ratio of WT splicing to exon truncation, consistent with NMD degradation of the aberrant transcript.
I) Manual patch-clamp of the WT and variant iPSC-CMs was performed to test the effect of aberrant splicing on protein function. Sodium currents were abrogated in the presence of the variant compared to WT, consistent with haploinsufficiency from loss-of-splicing in panel F.

We have updated the Results:

"Cryptic splicing effects of missense variants. Missense variants that disrupt gene function and lead to disease are usually presumed to disrupt protein function. Functional assays of missense variants are often performed using cDNA, which may obscure missense variant effects on splicing (Figure 6A).[63] We hypothesized that some *SCN5A* missense variants may cause Mendelian disorders such as BrS through an aberrant splicing mechanism rather than isolated disruption of protein function. In iPSC-CMs, we recovered determinate data for 48 missense variants; 28 were listed as VUS in ClinVar, and 20 were identified prospectively with high SpliceAI scores (>0.8). Of these, we identified 18 splice-altering missense variants, created by 20 unique splice-altering single nucleotide variants. These variants were distributed throughout the protein, but often clustered in hotspots near exon boundaries. To explore differences in missense variant splicing and protein function in cDNA and endogenous assays, we studied the clinically relevant missense variant c.4220C>G/p.A1407G. This variant was reported in a patient undergoing genetic evaluation for *SCN5A*-related disease in ClinVar and disrupted splicing in the ParSE-seq assay by causing a large out of-frame exon truncation event (Figure 6B-C). We then performed cDNA-based automated patch clamping experiments on this variant using HEK293 cells stably expressing *SCN5A* cDNA (Figure 6C and 6D). The variant had near-normal electrophysiologic function in this assay, with a normalized peak current density of 121.9±9.1% (n=46) of WT (Figure 6C and 6D). In addition to peak current, we also studied additional electrophysiological functions of voltages of half-activation, voltages of half-inactivation, recovery from inactivation, time constant τ of inactivation, and late current (Supplemental Figure XIV and Supplemental Table VII). We did not observe large differences in these parameters, except a minor left-shift of activation for p.A1407G and a right-shift of inactivation for p.A1407G, which are both predicted to cause slight gain-of-function. We also characterized a second missense variant, c.3392C>T/p.T1131I, which disrupted splicing in the ParSE-seq assay but had near-normal electrophysiological functions in the cDNA assay (Supplemental Figure XIV and Supplemental Table VIII).

Given the discrepancy in *in vitro* assays, we chose to further study the effect on splicing and protein function by introducing the c.4220C>G/p.A1407G variant at the endogenous locus in iPSC-CMs[64]. Using CRISPR-Cas9, we generated a heterozygous edit of c.4220C>G in a population control iPSC line (Figure 6E). Alongside the WT population control, both lines were differentiated into iPSC-CMs using a chemical differentiation method and then studied at the RNA level (RNA-seq) and protein level (patch-clamp; Figure 6F). To avoid confounding by nonsense-mediated decay (NMD) in the endogenous locus of heterozygous iPSC-CMs[65,66], we treated both isogenic lines with the NMD inhibitor cycloheximide (CHX) alongside a vehicle control (DMSO). We performed RNA-seq and observed reads corresponding to the ParSE-seq exon truncation event in the variant line treated with vehicle control (Figure 6H). The aberrant reads were increased after treatment with CHX (consistent with NMD degradation of out-of-frame transcript; Figure 6H). This truncation event was not observed in isogenic control lines treated with either DMSO or CHX (Figure 6H). To test whether variant-induced aberrant splicing affected protein-level function, isogenic pairs of iPSC-CMs were studied by patch clamping to measure sodium channel current (Figures 6I). We observed a decrease in sodium current across a range of voltages in the c.4220C>G line compared to the isogenic control, confirming the hallmark loss-of-function phenotype for *SCN5A*-linked BrS[67,68] (Figure 6I).

Thus, ParSE-seq can help identify a class of missense, splice-altering variants for which cDNA-based assays of protein function yield incorrect conclusions about variant pathogenicity. This result highlights that for missense variants, ParSE-seq can be used to complement traditional cDNA-based assays of protein function."

We have updated the Discussion:

"Functional assays of missense variants are often performed using cDNA. As cDNA contains only exons without intervening intronic sequence, any exonic variant effect on splicing will not be annotated (exon skipping and/or cryptic splice site activation causing exon truncation). Therefore, current cDNA assays such as patch clamp electrophysiology can mis-annotate the molecular impact of a splice-altering variant."

We have removed the *in silico* missense prediction analysis from the manuscript. As the reviewer notes, this analysis was complicated to understand and only tangentially relevant to the argument of the paper.

We have updated the Limitations:

"Although we validate two ParSE-seq splice-altering variants by CRISPR editing of the iPSC-CMs, most variants were tested only in multiplexed minigene assays."

Comment 12: In Fig. 7, the authors study a single mutant in the endogenous SCN5A gene in iPSC-CM using genome editing. They show a strong effect in patch-clamp measurements, but only a very weak accumulation of the predicted variant splice isoform in the heterozygous iPSCs – how do these observations match together? The authors should also clarify how common this mutation is in patients to allow the readers to better judge the significance of their result. Similarly, the prevalence should be provided for all disease-associated mutations considered in this work.

Response: We agree that the contrast of aberrant transcript in the iPSC-CMs and protein function may seem discordant, yet we and others have reported that this result can occur, e.g., due to Nonsense Mediated Decay of the transcript at the endogenous locus (PMID: 37164047, 34906502). We now study two mutations in CRISPR-edited iPSC-CMs at the endogenous locus. For both lines there are substantially more aberrantly spliced transcripts by RNA-seq when the lines were incubated with the NMD inhibitor cycloheximide than at baseline. This supports a model where the aberrantly spliced product is being formed, but then degraded by NMD due to a frameshift and premature truncation of the transcript.

As stated in Comment 5, we are unable to quantify disease prevalence for all variants in this study due to lack of affected patient counts in ClinVar, and limit this analysis to those included in PMID: 32893267. These counts are now part of Supplemental Table III.

Comment 13: Representations of the text and figures need to be further improved. Often the figure legend only repeats what is stated in the text without providing necessary details on what is shown and how these figures/results were obtained. Further examples below:
- The Abstract contains a lot of technical terminology and should be formulated in more general terms for a journal with a broad readership like Nature Communications.
- Fig. 1C, for example, lacks a proper legend
- Figure 6: The small graph on the bottom right of Fig6A requires axis labels and some kind of description / attribution in the figure legend. It would also be interesting to e.g. color-mark the data points in Fig6E that correspond to the two SNPs analyzed in C and D, if they are included here.

Response: We have updated the abstract as suggested:

Interpreting the clinical significance of putative splice-altering variants outside canonical splice sites remains difficult without time-intensive experimental studies. To address this, we developed Parallel Splice Effect Sequencing (ParSE-seq), a multiplexed assay to quantify variant effects on RNA splicing. We first applied this technique to study hundreds of variants in the arrhythmia-associated gene *SCN5A*. Variants were studied in ‘minigene’ plasmids with molecular barcodes to allow pooled quantification. Experiments were performed in two cell types, including disease-relevant induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). The assay strongly separated known control variants from ClinVar, enabling quantitative calibration of the ParSE-seq assay. Using these evidence strengths and experimental data, we reclassified 29 of 34 variants with conflicting interpretations and 11 of 42 variants of uncertain significance. In addition to intronic variants, we show that many synonymous and missense variants disrupted RNA splicing. Two splice-altering variants in the assay also disrupted splicing and sodium current when introduced into iPSC-CMs by CRISPR-Cas9 editing. ParSE-seq provides high-throughput experimental data for RNA-splicing to support precision medicine efforts and can be readily adopted to other loss-of-function genotype-phenotype relationships.

Figure legend 1C now reads:

C) Lollipop diagram of ClinVar reported *SCN5A* splice-altering variant locations. Green track shows *SCN5A* exons (rectangles) and introns (narrow line). Most Pathogenic (red) and Likely Pathogenic (orange) variants are located near the canonical splice sites, and are distributed throughout the gene product. There is only one Variant of Uncertain Significance (yellow) and four conflicting interpretation variants (CI; gray) that are annotated as ‘splice-altering’.

Figure legend 6A now reads:

Diagram showing that cDNA-based assays do not account for splice-altering variant effects. Left: Schematic of genomic locus with large intronic sequences; Right: cDNA-based sequence without introns used in many *SCN5A* functional assays. Alternating exons are annotated in green and blue.

Reviewer #4

The manuscript entitled “ParSE-seq: A Calibrated Multiplexed Assay to Facilitate the Clinical Classification of Putative Splice-altering Variants” report a very powerful use of biotechnology to understanding the consequences of genetic mutations on the function, in this case, of ion channel. But of course it can be applied to any other functional proteins. In particular its usefulness is in regards to mutations found in splice-altering variants, that are usually neglected. Due to my background, I limited my revision to the electrophysiology section of the study, for which I have few observations.

Comment 1: Paragraph “Cryptic splicing effects of missense variants”, lines 524-526. The Authors stated that the two VUS had near-normal electrophysiologic function, but they limited their observation to the peak current density. I ask to investigate also other electrophysiological properties, as it is known that the kinetics of the channels may be affected by mutations, and the voltage dependence as well. Working with automated patch-clamp may be useful for information coming from a simple Current-voltage relationship protocol (form which, it is possible to have at least the activation curve of the channel, and also evaluate the kinetic of the fast inactivation). I am sure that the Authors are aware that the Nanion Syncropatch can also be implemented with protocol for the study of the availability curve. In this sense, I also ask to have example of typical current traces of the NaV1.5 p.T1131I and p:A1407G in Figure 6.

Response: We performed additional SyncroPatch experiments to record the requested electrophysiological properties. We present an updated Figure 6 that includes typical current traces for WT and p.A1407G. As part of our response to reviewers 1-3, we included new iPSC-CM data to fully explore the c.4220C>G/p.A1407G variant, and now refer to p.T1131I in the Supplement. In a new Supplemental Figure XIV we show additional variant properties, including voltage dependence of activation and inactivation, and recovery from inactivation. We added a new Supplemental Table VIII quantifying numbers of cells and normalized, mean data.

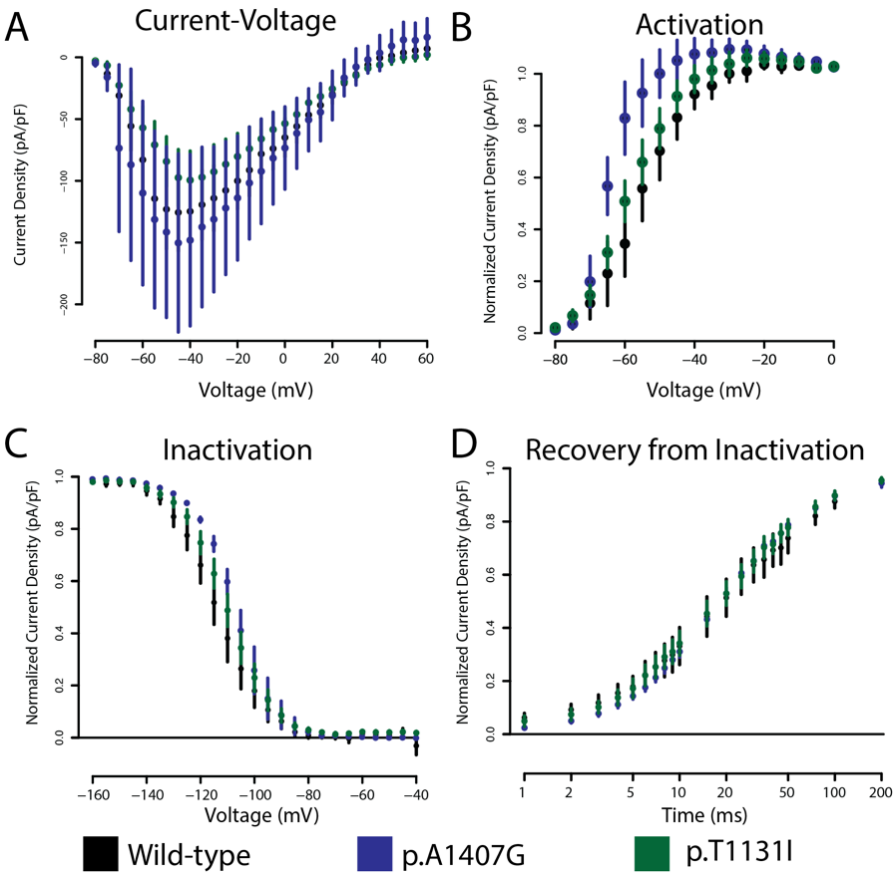
We have updated the results to read:

“In addition to peak current, we also studied additional electrophysiological functions of voltages of half-activation, voltages of half-inactivation, recovery from inactivation, time constant τ of inactivation, and late current (Supplemental Figure XIV and Supplemental Table VII). We did not observe large differences in these parameters, except a minor left-shift of activation for p.A1407G and a right-shift of inactivation for p.A1407G, which are both predicted to cause slight gain-of-function. We also characterized a second missense variant, c.3392C>T/p.T1131I, which disrupted splicing in the ParSE-seq assay but had near-normal electrophysiological functions in the cDNA assay (Supplemental Figure XIV and Supplemental Table VIII)”.

Supplemental Table VIII. Normalized electrophysiologic Data for two $Na_V1.5$ variants. Standard error of the mean is shown in parentheses for raw values.

Variant	Normalized Peak Density	Cells	
A1407G	121.9 (9.1)	46	
T1131I	94 (7.3)	67	
	Mean V_act	**Cells**	**Normalized Mean**
A1407G	-64 (3.7)	5	-20

T1131I	-58 (2.3)	16	-14
	Mean Tau_inactivation	**Cells**	**Normalized Mean**
A1407G	1.431 (0.069)	45	-0.343
T1131I	1.069 (0.033)	50	-0.705
	Mean V_inact	**Cells**	**Normalized Mean**
A1407G	-107.1 (2.4)	5	-8.7
T1131I	-110.2 (2.0)	20	-11.8
	Recovery from Inact 50	**Cells**	**Normalized Mean**
A1407G	20.4 (1.0)	5	9
T1131I	22.3 (2.6)	18	10.9
	Late Current Ratio (50 ms)	**Cells**	
A1407G	0.0038 (0.00009)	15	
T1131I	0.0017 (0.00009)	21	



Supplemental Figure XIV. Electrophysiological parameters of suspected splice-altering missense variants in a cDNA high-throughput automated patch-clamp assay.
A) Current-Voltage (IV) curve for two splice-altering missense variants. Distributions of currents overlap between WT (black), p.A1407G (blue), and p.T1131 (green) across the tested range of voltages.
B) Activation curve for the WT $Na_V1.5$ and two missense variants. A left-shift of activation is observed for p.A1407G (phenotypic gain-of-function).
C) Inactivation curve for WT $Na_V1.5$ and two missense variants. A right-shift of inactivation is observed for p.A1407G (phenotypic gain-of-function).
D) Recovery from inactivation for WT $Na_V1.5$ and two missense variants.

Comment 2: Figure 7, panel F, G and H. Comparing the current amplitude of the traces in panel F with the Current-Voltage relationship (IV) in panel H (and doing the same for the Panel G), it emerged that the capacitance of the cells patched should be about 60 pF and 83 pF, respectively. This is quite uncommon dealing with hiPSCs-CM, where the capacitance is usually 30-40 pF (see for references PMID 27672365 and 23029342). Would it be possible that instead of single cells, small group of cells was patched? Moreover, looking on the IV reported in panel H, the WT channels peaks at -30 mV (uncommon, it is known to peak at -20 mV). This result could be possibly due to a poor compensation since the current measured are extremely large (see panel F, despite in in this case the compensation is good), and maybe the capacitance large as well. On the other hand, considering panel G, in this case the traces are not well compensated and the voltage is not well controlled, despite in the IV peaks at -20 mV. I therefore suggest to check the experiments lowering the sodium concentration in the extracellular solution (here is 50 mM for the manual patch clamp, the suggestion is to decrease at 20 mM).

Re: iPSC-CM cell size:
The cell size of iPSC-CMs is somewhat variable and likely depends on exact differentiation conditions. In addition to the two papers that the reviewer mentioned above, others have reported a wide range of cell sizes: 88.7 +/- 5 pF (PMID: 21890694) and 17+/-1 pF (PMID: 26429802). In our study, the cell sizes of iPSC-CMs at Day 30-40 varied from 33.4 to 82.5 pF, with an average capacitance of 56.2 +/- 4.8 pF (WT), 44.3 +/-1.7 pF (c.1891-5G>C variant), and 45.9 +/-1.8 pF (c.4220G>C variant).

Re: voltage control:
There is also a slight range of reported values for what voltage results in peak $I_{Na.}$. A computational study in combination of experimental iPSC-CM sodium current data from different laboratories shows that peak I_{Na} reached at -20 or -30 mV (Figure 3 from PMID 31278749) at different external sodium concentrations (50 or 20 mM). The current manuscript and previous work (Figure 2 from PMID: 36524479) studied I_{Na} in iPSC-CMs at ~Day 35 both measured peak I_{Na} at -30 mV at external sodium concentration of 50 mM.

Re: lowering of sodium from 50 mM to 20 mM:
We appreciate the suggestion to change sodium concentrations. As the reviewer is aware, larger external sodium concentrations result in larger sodium currents. We chose an external sodium concentration (50 mM) that gave us medium to large sodium currents at baseline. Using this concentration, we had a good experimental ability to detect reductions in current due to loss-of-function splice-altering mutations. Lowering the sodium concentration to 20 mM would result in smaller overall currents, which would certainly help keep the cells in voltage control. However, 20 mM sodium would reduce our dynamic range to observe the effects of loss-of-

function mutations. Therefore, we chose to perform our experiments with an external concentration of 50 mM.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
While AI has progressed and its predictive accuracy has improved, it cannot fully explain all splicing mechanisms, and high-throughput experimental systems are still needed. The authors' method has the weakness of being time-consuming in preparing mini-genes, but I believe it has sufficient performance to complement these AI predictions. I also think it's good that there is an example of SCN5A. In the future, it would be great if more data on the genetic variant – aberrant splicing correlation would be accumulated using this system so that AI can use the data for training.
Regarding my concerns, although there were some comments which was difficult to answer, I think the authors responded as much as possible.
Going forward, I hope they will use this system to comprehensively and thoroughly examine other genes associated with disease as well.

Reviewer #2:
Remarks to the Author:
We thank the authors for the care and clarity of their response to Reviewer comments. No further comments

Reviewer #3:
Remarks to the Author:
The authors addressed our comments satisfactorily. We congratulate them to their excellent work!

Reviewer #5:
Remarks to the Author:
In this work, the authors introduce a novel multiplexed splicing assay, which appears to be a powerful approach for screening splice-altered protein variants amongst hundreds of candidates. The authors used the SCN5A sodium ion channel as an example and also evaluated the splicing protein's functional outcomes by conducting electrophysiological studies. They assessed the function of selected splicing variants of the channel expressed in HEK cells and iPSC-derived cardiomyocytes. Judging from the electrophysiological experiments, I can appreciate that the tests were performed

accurately, and the authors made efforts to implement most of the commonly used electrophysiological protocols to characterize the Nav channel function. Additionally, they successfully integrated the splice variant of the channel into iPSC-derived cardiomyocytes using the CRISPR-Cas9 editing approach.

The study's limitation lies in testing only two slicing variants to assess their function. While the authors explained the practical reasons behind this choice, it's important to note that these selected variants may not accurately represent the typical outcomes of disrupted function. The authors emphasized the time-consuming and expensive nature of these tests, but they should also consider a more comprehensive discussion of the potential functional consequences of splice alterations based on the limitations of the experimental data.

Additionally, there are some specific and technical issues that need to be addressed.

1. The biophysical parameters of Nav channel function added to the revised manuscript as requested provide essential information for the changed splicing variants. However, these have been shown only for HEK cells but not for the splicing channel function incorporated in iPCS-derived cardiomyocytes. These parameters are essential to show for the iPSC-derived cardiomyocytes.

2. Further to the above, the reduced current density might not necessarily indicate a change in channel function but a decreased expression of functional Nav. Therefore, the altered Nav function in iPCS-derived cardiomyocytes should be confirmed by presenting other parameters of the channel function, similar to what was done in HEK cells or, at the very least, confirming the protein expression level.

3. In Supplemental Figure XIV, it seems that a left-shifted activation for p.A1407G in HEK cells is not ‘minor’ – it could be in the 5-10 mV range. Please provide the P value. Such a shift for Nav channels can result in a substantial gain-of-function and cell hyperexcitability and strengthen the functional consequences of splicing variants.

4. The reported variability in the passive parameters of the iPSC-derived cardiomyocytes is not somewhat surprising, as differentiation of iPSCs is largely variable, especially in higher confluence cultures (up to 80% herein). It is important to provide not only capacitance but also other parameters, such as membrane resistance and membrane potential of individual cells tested, to understand the functional maturation of the cells and the level of ion channel conductance.

5. More details are needed for the methodology of electrophysiological tests. Was the series resistance controlled throughout the recordings? Were the protocols applied

repeatedly to individual cells for testing various parameters? How many trials were conducted, and were trials averaged if recorded from the same cells?

6. In Figure 6D, please add the P value to show the statistical difference between both groups.

Reviewer #1

While AI has progressed and its predictive accuracy has improved, it cannot fully explain all splicing mechanisms, and high-throughput experimental systems are still needed. The authors' method has the weakness of being time-consuming in preparing mini-genes, but I believe it has sufficient performance to complement these AI predictions. I also think it's good that there is an example of SCN5A. In the future, it would be great if more data on the genetic variant – aberrant splicing correlation would be accumulated using this system so that AI can use the data for training.
Regarding my concerns, although there were some comments which was difficult to answer, I think the authors responded as much as possible.
Going forward, I hope they will use this system to comprehensively and thoroughly examine other genes associated with disease as well.

Response: We are pleased to incorporate these new AI data and to provide a method for generating new experimental data for AI training. We are looking forward to studying additional genes in the future.

Reviewer #2

We thank the authors for the care and clarity of their response to Reviewer comments.
No further comments

Response: Thank you.

Reviewer #3

The authors addressed our comments satisfactorily. We congratulate them to their excellent work!

Response: Thank you.

Reviewer #5

In this work, the authors introduce a novel multiplexed splicing assay, which appears to be a powerful approach for screening splice-altered protein variants amongst hundreds of candidates. The authors used the SCN5A sodium ion channel as an example and also evaluated the splicing protein's functional outcomes by conducting electrophysiological studies. They assessed the function of selected splicing variants of the channel expressed in HEK cells and iPSC-derived cardiomyocytes. Judging from the electrophysiological experiments, I can appreciate that the tests were performed accurately, and the authors made efforts to implement most of the commonly used electrophysiological protocols to characterize the Nav channel function. Additionally, they successfully integrated the splice variant of the channel into iPSC-derived cardiomyocytes using the CRISPR-Cas9 editing approach.

The study's limitation lies in testing only two slicing variants to assess their function. While the authors explained the practical reasons behind this choice, it's important to note that these selected variants may not accurately represent the typical outcomes of disrupted function. The

authors emphasized the time-consuming and expensive nature of these tests, but they should also consider a more comprehensive discussion of the potential functional consequences of splice alterations based on the limitations of the experimental data.

Response: We agree that our results suggest further biophysical experiments to investigate aberrant splicing consequences; however, a primary focus of the ParSE-seq method was for actionable clinical classification. Accordingly, we have updated the discussion:

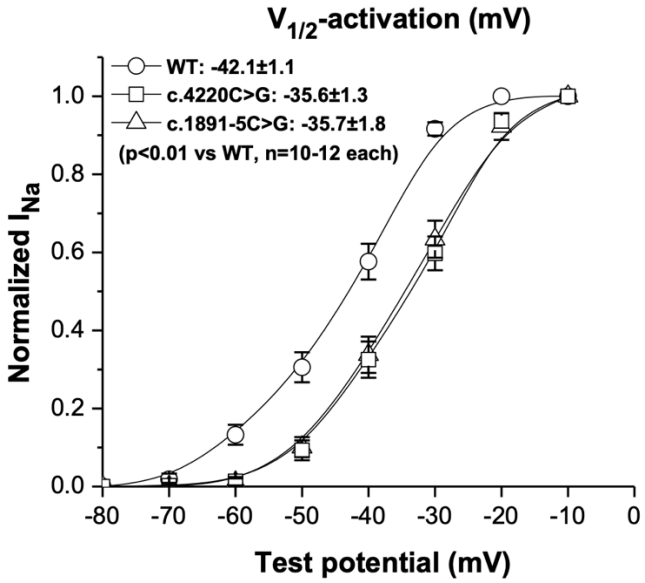
"Although we validate two ParSE-seq splice-altering variants by CRISPR editing of the iPSC-CMs, most variants were tested only in multiplexed minigene assays. While we anticipate most splice-altering variants to result in loss-of-function ($Na_V1.5$ peak current abrogation for *SCN5A* variants), there may be alternative mechanisms revealed by functional assessment of the CRISPR-edited iPSC-CM model. For example, while ParSE-seq quantifies broad molecular impacts such as exon skipping, exon truncation, and intron retention, it is possible that some splicing abnormalities may not have a detrimental effect, or otherwise altered effect, on downstream protein function (protein tolerant in-frame insertion/deletions)."

Additionally, there are some specific and technical issues that need to be addressed.

Comment 1: The biophysical parameters of Nav channel function added to the revised manuscript as requested provide essential information for the changed splicing variants. However, these have been shown only for HEK cells but not for the splicing channel function incorporated in iPCS-derived cardiomyocytes. These parameters are essential to show for the iPSC-derived cardiomyocytes.

Response: In the HEK cell studies, the variants were introduced as cDNA and therefore not subject to aberrant splicing, and had near-wildtype-like peak current. Therefore, the additional variant electrophysiological parameters requested by Reviewer 4 could readily be measured. In the iPSC-CM experiments, the variants were introduced in the heterozygous state at the endogenous genomic locus and therefore could affect both RNA splicing and protein function. For both studied variants, we observed aberrant splicing of the mutant allele, and degradation of the mutant transcript by Nonsense Mediated Decay, which we demonstrated by adding the NMD inhibitor cycloheximide. As a downstream consequence of this splicing aberration, we observed reduced peak sodium currents.

In addition to the peak current quantification and IV curves already in the manuscript, we now include the new figure below showing activation curves in iPSC-CMs and quantify the voltage of half activation. Note however, that this represents functional interaction of both the wild-type and variant allele.



Supplemental Figure XV. Voltages of half activation of iPSC-CM models.

We have updated the results:

"We observed a decrease in peak sodium current across a range of voltages in the c.4220C>G line compared to the isogenic control, confirming the hallmark loss-of-function phenotype for *SCN5A*-linked BrS[68,69] (Figure 6I). We also determined voltages of half-activation for each iPSC-CM line, and observed a statistically significant decrease in the mean voltage of half-activation for c.4220C>G, consistent with loss-of-function (-35.6 vs -42.1 mV, $p < 0.01$, N=10-12; Supplemental Figure XV)."

We did not perform an extensive characterization of additional properties in the iPSC-CMs, because these measurements would be mainly measuring the wildtype allele, not the mis-spliced and mostly NMD-degraded mutant allele. In the HEK cell experiments, we were able to directly measure the electrophysiological properties mutant channel by itself from a pre-spliced cDNA in a hemizygous system. However, the mutant transcripts are mostly degraded in the endogenous context due to a splicing defect and NMD, as we demonstrated in the iPSC-CMs. This result emphases the potential inconsistency of assays that use cDNA versus endogenous locus for variants that affect RNA splicing.

Comment 2: Further to the above, the reduced current density might not necessarily indicate a change in channel function but a decreased expression of functional Nav. Therefore, the altered Nav function in iPCS-derived cardiomyocytes should be confirmed by presenting other parameters of the channel function, similar to what was done in HEK cells or, at the very least, confirming the protein expression level.

Response:

We agree that the reduced current density in the iPSC-CMs does not reflect a change in channel function but a result of decreased expression of the mutant allele due to splicing defects and NMD (best illustrated by drug response in Figure 6H). Functionally, this expression change manifests with the near-normal peak currents of the mutants in HEK cells with pre-

spliced cDNA, in contrast to the reduced peak currents in iPSC-CMs via NMD degradation (in the endogenous splicing context).

We have updated the Discussion to clarify this point:

“Loss of function variants in ion channel genes have been classified into four categories: Class I (RNA degradation or reduced expression), Class II (diminished trafficking), Class III (changes in gating), or Class IV (changes in ion permeability) (PMID: 15192037). Traditionally, many missense variants in *SCN5A* have acted through a Class III or Class IV mechanism (PMID: 29728395). However, our data show that the missense variant c.4220C>G/p.A1407G appears to mainly act through a Class I mechanism.”

Comment 3: In Supplemental Figure XIV, it seems that a left-shifted activation for p.A1407G in HEK cells is not ‘minor’ – it could be in the 5-10 mV range. Please provide the P value. Such a shift for Nav channels can result in a substantial gain-of-function and cell hyperexcitability and strengthen the functional consequences of splicing variants.

Response:

We have removed the word “minor” to describe this shift in the activation curve. We also now provide P values for this difference compared to wildtype.

We have updated the results:

“We did not observe large differences in these parameters, except for a left-shift of activation for p.A1407G (P = 0.09, two-tailed T test) and a right-shift of inactivation for p.A1407G (P = 0.069, two-tailed T test), which are both predicted to cause gain-of-function (Supplemental Figure IVX and Supplemental Table XIII).”

Comment 4: The reported variability in the passive parameters of the iPSC-derived cardiomyocytes is not somewhat surprising, as differentiation of iPSCs is largely variable, especially in higher confluence cultures (up to 80% herein). It is important to provide not only capacitance but also other parameters, such as membrane resistance and membrane potential of individual cells tested, to understand the functional maturation of the cells and the level of ion channel conductance.

Response:

As the reviewer notes, the cell size and maturity of iPSC-CMs is somewhat variable, and the measured properties will therefore have some variability. However, they are consistent with other iPSC-CMs studied by our lab (PMID: 29563327, 24895457, and 36524479) and other groups (PMID: 21240260, 23015789, 21890694).

We have updated the Methods:

“The average capacitances of the iPSC-CMs were 56.9 ± 4.8 pF (WT), 50.6 ±2.5 pF (c.1891-5G>C), and 45.9 ±0.8 pF (c.4220G>C). The average membrane resistances of the iPSC-CMs were 1.54±0.09 GΩ (WT), 1.53±0.08GΩ (c.1891-5G>C variant), and 1.55±0.1GΩ (c.4220G>C variant). These parameters were not statistically significantly different from each other (p>0.05).

We did not measure membrane potentials during the sodium current (INa) measurements under modified experimental conditions (see below). However differentiated iPSC-CMs from the same population control iPSC line studied with physiological intra- and extracellular solutions had potentials ranging from -75. to -90 mV, with an average potential of -82.2 ± 1.2 mV."

We have updated the Results:

"In addition to peak current density, we also provide membrane resistance for the current CRISPR-edited iPSC-CM lines and reference membrane potential data for our population control line (Supplemental Table IX; PMID: 36524479)".

We now include Supplemental Table IX:

Membrane potential (mV) †														
Cell#	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Summary (mV)**
WT	-75	-77.6	-81.3	-81.4	-84.5	-80.4	-82.8	-90	-87.3	-78.6	-86.3	-84.6	-78.6	-82.2±1.2
Capacitance (pF)														
Cell#	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Summary (pF) ***
WT	46.3	42.6	38.9	72.3	41.5	56.2	50.6	70.5	67.8	82.5				56.9±4.8
c.4220c>G	47.2	48.3	45.6	46.8	43.8	44.5	47.3	48.9	46.2	40.2				45.9±0.8
c.1891-5C>G	47.3	50.3	51.3	46.8	41.6	44.5	67.4	68.2	46.5	43.7	46.2	53.6		50.6±2.5
Membrane resistance (GΩ)														
Cell#	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Summary (GΩ) ***
WT	1	1.2	1.8	1.4	1.6	1.5	2	1.6	1.7	1.6				1.54±0.09
c.4220c>G	1.6	1	1.4	1.8	1.9	2	1.7	1.6	1.3	1.2				1.55±0.1
c.1891-5C>G	1.1	1	1.4	1.5	1.8	2	1.6	1.4	1.9	1.4	1.8	1.5		1.53±0.08

Supplemental Table IX. Passive parameters of iPSC-CM manual patch-clamp experiments. Recorded parameters are presented for each cell. Cell capacitance and membrane resistance are provided for all single cells used in this study. Since the current study focused on INa measurements under modified experimental conditions (see below), using current-clamp mode was not able to accurately monitor membrane potentials. †Membrane potential from previously published data by our laboratory (PMID: 36524479). *****p > 0.05, no statistically significant difference among groups.

Recording of the passive parameter of membrane potential was limited in the current experimental setting. To improve voltage control during sodium current recordings, we used a modified K^+-/Ca^{2+}-free extracellular solution with a lowered external sodium concentration of 50 mM. Under these experimental conditions, it was not possible to accurately monitor the membrane potentials (MPs) of iPSC-CMs by using current-clamp mode. In our previous studies (PMID: 29563327, 24895457, and 36524479), however, an external potassium concentration of 4 mM (physiological concentration) in Tyrode's solution was used for action potential (AP) recording experiments. In the relatively "mature" ventricular iPSC-CMs used (Day 30-40), we observed that the MP ranged from ~ -75 to ~ -90 mV. In our hands, these cells are stable and can allow for prolonged experiments. Here we list our previous MP data in wild-type iPSC-CMs (-82.2±1.2 mV, n=13), which is consistent with reports from other groups (PMID: 21240260, 23015789, 21890694).

Comment 5: More details are needed for the methodology of electrophysiological tests. Was the series resistance controlled throughout the recordings? Were the protocols applied repeatedly to individual cells for testing various parameters? How many trials were conducted, and were trials averaged if recorded from the same cells?

Response:

We have updated the methods:

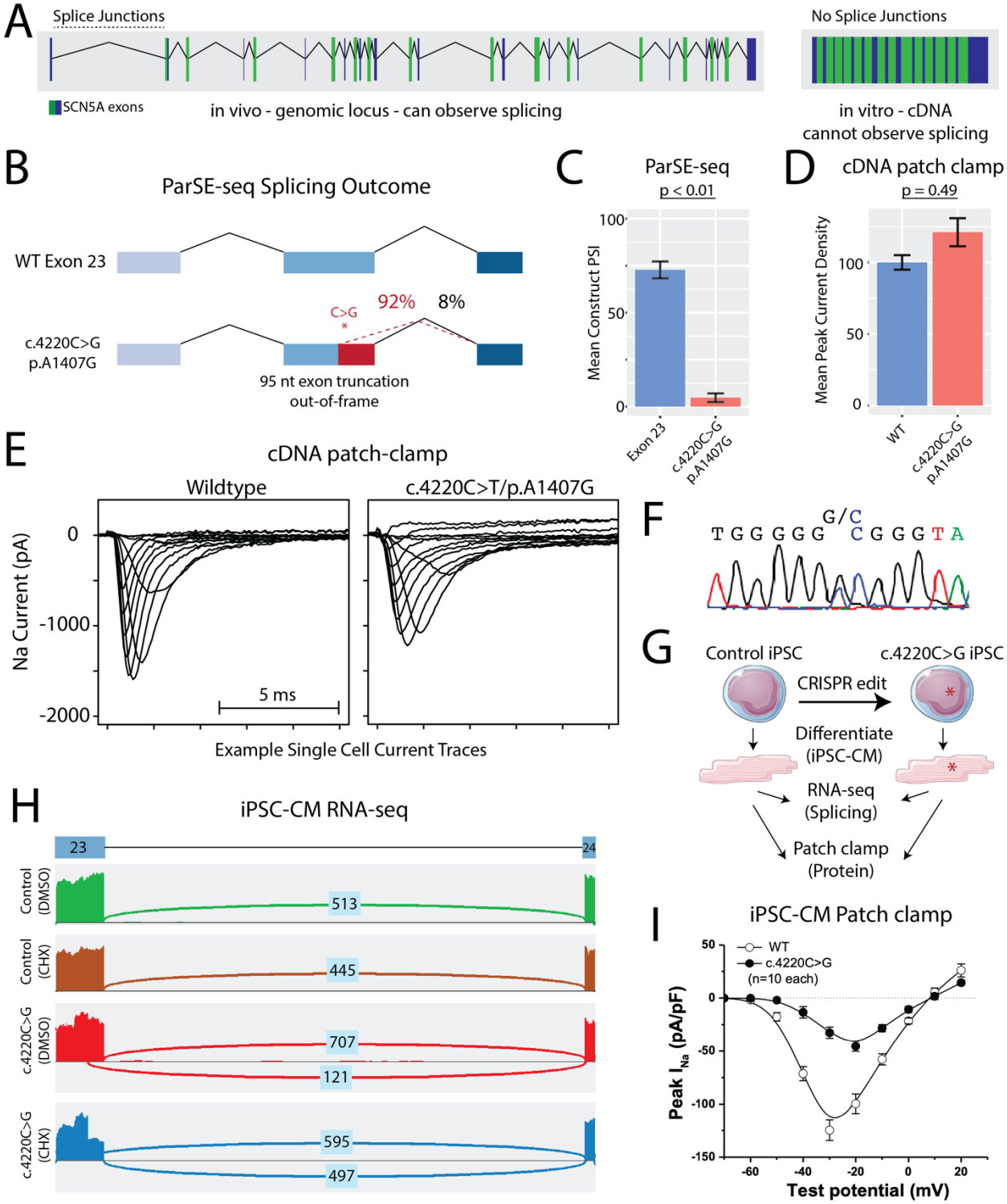
"In the SyncroPatch HEK293 and manual iPSC-CM patch experiments, the series resistance (*Rs*) was monitored using the Seal Resistance QC variable to achieve a range of 5-10 MΩ. Multiple protocols were applied sequentially to each cell, including current-voltage (IV) curves to measure INa activation, inactivation, recovery from inactivation, and late current protocols. A full description of this method has been published (PMID: 32533946). Two independent transfections were performed for Syncro Patch and manual experiments for each mutant, and data were averaged across all cells passing QC criteria as previously described (PMID: 32533946). In the manual iPSC-CM patch-clamp experiments, the series resistance (*Rs*) was monitored using *Seal Test* (Clampex 10.9 software) to achieve a range of 5-10 MΩ. Current-voltage curves were generated by repeated voltage changes to the same cells. Two trials were performed for each cell line and data was then averaged across all measured cells."

And:

"During manual patch-clamp experiments on iPSC-CMs, we selected iPSC-CMs at age of Day 30-40. During the experiments, membrane resistance (*R*m) was monitored throughout using the *Membrane Test* (Clampex 10.9). We first optimized the electrode capacitance compensation on the amplifier, performed following giga-seal formation and before achievement of the whole-cell configuration. In this way, the capacitive transients were completely and well-compensated by ~80% when whole-cell capacitance compensation was enabled. Next, we used *Seal Test* (Clampex 10.9) as an oscilloscope window for monitoring the current signal to achieve a reading close to 10 MΩ. Optimization of the capacitance compensation was an extremely important step for accurate Cm and *Rm* measurements in *Membrane Test*. To achieve high quality giga-seal formation before cell membrane break-in, we chose cells with giga-seal of 1-2 GΩ for the experiments."

Comment 6: In Figure 6D, please add the P value to show the statistical difference between both groups.

Response: We have updated the figure and legend accordingly:



And updated the relevant Figure Legend sections accordingly:

C) Quantification of mean canonical PSI among reads for the WT exon construct and variant construct. Error bar corresponds to standard error of the mean. P-values were calculated from a two-tailed t-test.
D) Quantification of sodium channel current densities for WT $Na_V1.5$ and variant $Na_V1.5$ using the SyncroPatch automated patch-clamping system (cDNA assay), in stably expressing HEK293 cells. Error bar corresponds to standard error of the mean. P-values were calculated from a two-tailed t-test.

Reviewers' Comments:

Reviewer #5:
Remarks to the Author:
Thanks to the authors for providing clarity, considering each of the comments raised, and adding more details to the text.
Congratulations on this nice paper!