An enhancer cluster controls gene activity and topology of the SCN5A-SCN10A locus in vivo

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Mutations and variations in and around SCN5A, encoding the major cardiac sodium channel, influence impulse conduction and are associated with a broad spectrum of arrhythmia disorders. Here, we identify an evolutionary conserved regulatory cluster with super enhancer characteristics downstream of SCN5A, which drives localized cardiac expression and contains conduction velocity-associated variants. We use genome editing to create a series of deletions in the mouse genome and show that the enhancer cluster controls the conformation of a >0.5 Mb genomic region harboring multiple interacting gene promoters and enhancers. We find that this cluster and its individual components are selectively required for cardiac Scn5a expression, normal cardiac conduction and normal embryonic development. Our studies reveal physiological roles of an enhancer cluster in the SCN5A-SCN10A locus, show that it controls the chromatin architecture of the locus and Scn5a expression, and suggest that genetic variants affecting its activity may influence cardiac function.
he SCN5A gene encodes the alpha subunit of the major voltage-gated sodium channel Na,1.5. Cardiac sodium channels are essential for fast conduction of electrical impulses through myocardium and as such play an important role in excitation and contraction of the heart. Deletions, and gain- or loss-of-function mutations in SCN5A are associated with a spectrum of human cardiac conduction diseases including bradycardia, atrial fibrillation, and Brugada syndrome. Only in about 25% of the Brugada syndrome patients, however, a mutation is found in the coding sequence of SCN5A, indicating a role of other genes or of other mechanisms affecting SCN5A expression1,2. Genome-wide association studies (GWAS) have identified genetic variants in the SCN5A-SCN10A locus associated with conduction velocity as indicated by the PR interval and QRS duration of the ECG3–5. Interestingly, an unexpectedly large cumulative effect of variants in the SCN5A-SCN10A and HEY2 loci on Brugada syndrome susceptibility was discovered6. The majority of disease-associated genetic variants are found in non-coding regulatory DNA regions that control gene expression7.

The spatial and temporal expression patterns of genes are controlled by regulatory elements (REs), which include enhancers8. REs are occupied by lineage-specific and general transcription factors and interact with promoters of their target genes in order to regulate transcription. Distinct epigenetic signatures have identified over a million of putative REs in different human and mouse cell types8–11. REs and their target genes are usually present within the same topologically associated domains (TADs)8,12–14. The expression of most genes is regulated by multiple REs, and REs may control multiple target genes. The regulatory relationships among active REs is complex (e.g. additive, synergistic), condition-specific (e.g. cell-type, developmental stage) and has not been defined for the vast majority of genes and conditions8,15. Particular epigenetic signatures and activities indicate the existence of large densely clustered REs, dubbed “super enhancers”, which are often found near genes regulating cell identity16,17. Genes regulated by super enhancers are sensitive to perturbations leading to large phenotypic changes. While RE function has been extensively studied, insight into the in vivo physiological role and function of REs in mammals is limited8,18.

Trait- or disease-associated variants supposedly alter transcription factor binding sites which changes the activity of REs, thereby modulate target gene expression15. Nevertheless, in vivo functional variants are rarely identified and the underlying mechanism usually remains obscure19,20. Moreover, because REs can be located kilobases (Kb) away from their target genes, ignoring genes and REs in between, it remains difficult to predict which genes are influenced by a particular variant RE. Locus-specific and genome-wide physical proximity maps have been generated through chromosome conformation capture technologies, which has provided valuable information regarding chromatin topology and possible interactions between putative regulatory sequences and promoters12,21,22. These maps are mostly derived from cultured, non-cardiac cells, and most datasets are of limited resolution. Recently, promoter-capture Hi-C maps of cardiomyocytes derived from human stem cells have been generated with greatly increased power to detect interactions involving promoter sequences in a cardiac relevant cell type23,24. However, close proximity by itself does not predict whether a regulatory sequence regulates a particular gene34, and the number of in vivo proven target genes of tissue-specific REs in mammals is still limited.

Previously, we and others have identified several cardiomyocyte-specific REs in the human SCN5A-SCN10A locus that are capable of driving reporter gene expression in the embryonic mouse heart in patterns resembling that of Scn5a1,25–27. An RE in an intron of SCN10A (RE1) was found to harbor a common genetic variant associated with PR interval3 that disrupts a T-box factor binding site, which was associated with decreased SCN5A expression levels in human hearts27. RE5 was identified in an intron of SCN5A and RE6 downstream of SCN5A. RE6 contains genetic variants associated with PR interval and QRS duration3,4.

Here, we focus on the function of putative REs downstream of SCN5A that are part of a large cluster showing the hallmarks of a “super enhancer”16,17. Using genome editing technologies we delete different portions of the enhancer cluster from the mouse genome. We demonstrate that deletion of the enhancer cluster and its individual components cause a decrease in Scn5a expression and conduction slowing. Furthermore, deletion of the enhancer cluster causes a marked change in the chromatin architecture of the locus. Our studies reveal the regulatory function of an enhancer cluster in the SCN5A-SCN10A locus, and further suggest the relation between genetic variants found in non-coding regions in the SCN5A-SCN10A locus, RE function and the susceptibility to arrhythmias.

Results

Identification of a cardiac-specific SCN5A super enhancer. To determine the TADs boundaries of the SCN5A-SCN10A locus, we used available Hi-C data derived from lymphoblastoid cell line GM1287821,28. TADs are largely invariant between different cell types and conserved across species8,12. We found that SCN5A and SCN10A share the TAD with EXOG, SCN11A, and WDR48 (Fig. 1a). Enrichment of interactions within this region observed in promoter-capture Hi-C data of iPSC-derived cardiomyocytes,23 further supports the assignment of the TAD (Fig. 1a). The spatial organization of this TAD is conserved in between human (Fig. 1a) and mouse27. The activity of REs is largely limited to target genes that fall within the same TAD8, including SCN5A, SCN10A, EXOG, SCN11A, and WDR48.

GWAS have identified genetic variants in the SCN5A-SCN10A locus that modulate ECG parameters and contribute to conduction diseases. We plotted the –log(P value) < 0.05 of the genetic variants associated with PR interval and QRS duration3–5 in the SCN5A-SCN10A locus. Interestingly, variants significantly associated with PR interval were mainly located over SCN10A, whereas variants associated with QRS duration in left ventricular hypertrophy were distributed over SCN5A and SCN10A (Fig. 1a). We observed co-localization of QRS duration-associated variants with increasing statistical significance in the EXOG-SCN5A intergenic region (Fig. 1a). Based on EMERGE prediction29 (Supplementary Fig. 1 and Supplementary Table 1), the intergenic region is composed of four putative REs, which we named RE6-9 (Fig. 1b). Because of its size and its extensive association with H3K27ac30, the entire region has been classified5 as a super enhancer by the ENCODE project (Fig. 1c)4,25. Next, we examined conservation of the intergenic region RE6-9 between mouse and human. hRE6-9 covered 97.4% of span and 61.7% base pair alignment to the mouse genome. Based on a mouse cardiac-specific H3K27ac signature33 and EMERGE enhancer prediction29 (Supplementary Table 1), the four REs are also identified in the Exog-Scn5a intergenic region (Fig. 2a, b). Furthermore, the mouse RE6-9 (mRE6-9) is occupied by various cardiac transcription factors (e.g. Tbx3, Tbx5, Tbx20, Nkx2-5 and Gata4) and other epigenetic motifs and signatures.
Fig. 1 Identification of a cardiac-specific, multi-component ‘super enhancer’ in the human SCN5A-SCN10A locus. **a** Hi-C heatmap, visualized using HiGlass, from human lymphoblastoid line GM12878\(^{11}\) of the SCN5A TAD. -log(P values) of the genetic variants associated with PR interval\(^3,5\) and QRS duration\(^4\), human EMERGE track\(^29\) and CTCF ChIP human cardiac muscle cells are included of the SCN5A-SCN10A locus. **b** UCSC browser view of the intergenic region between SCN5A and EXOG, composed of four constituent regulatory elements (RE6-9) with “super enhancer” characteristics based on human EMERGE, H3K27ac ChIP human left ventricle and Mediator 1 (MED1) ChIP from iPSC-derived cardiomyocytes\(^32\). PR interval-associated and QRS duration-associated (-log(P value)) variants are shown in the enhancer cluster. **c** Dorsal view of E10.5 hearts containing human regulatory elements RE6\(^4\), 7 and 9\(^{25}\) in a Hsp68/LacZ enhancer reporter vector. Human RE6 (4/5) is active in the interventricular septum and 9 (6/8) shows in vivo activity in the interventricular septum and left ventricle of embryonic mouse heart. RE7 (1/3) displays only cardiac activity in the embryonic outflow tract. Black scale bar is 0.5 mm. LA left atrium, LV left ventricle, RA right atrium, RV right ventricle, AVC anterior ventricular canal
Fig. 2 Identification of a cardiac-specific, multi-component ‘super enhancer’ in the mouse Scn5a-Scn10a locus. a UCSC browser view of Scn5a-Scn10a locus with TAD obtained from Hi-C data on mouse embryonic stem cells (ESC), cardiomyocyte-specific H3K27ac ChIP33, mouse EMERGE29 and CTCF ChIP adult mouse heart. b UCSC browser view of the intergenic region between Scn5a and Exog, composed of four constituent regulatory elements (RE6-9) with ChIP-seq profiles of Tbx3 (black), Tbx5 (black), Tbx20 (black), Gata4 (black), Nkx2-5 (black), Pol2 (black), P300 (black) from the heart. H3K27ac (green) in cardiomyocytes and cerebellum, mouse and human EMERGE29 tracks (red) and conservation track GERP (bottom). c Dorsal view of mouse regulatory region RE6-9 in a Hsp68/LacZ enhancer reporter vector. Mouse RE6-9 (3/3) shows predominant activity in the interventricular septum and ventricles and to a lesser extent in the atria, in 2 of 3 E11.5 embryos. Black scale bars are 1 mm (embryo) and 0.5 mm (heart). d An example showing a zoomed-in view of predicted transcription factor binding sites Tbx5, Tbx20 and GATA4 in RE9 including sequence alignment between species and JASPAR consensus site. LA left atrium, LV left ventricle, RA right atrium, RV right ventricle, AVC anterior ventricular canal.
RE6-9 controls the chromatin architecture of the Scn5a-Scn10a locus. The three-dimensional organization of the Scn5a-Scn10a locus suggests that the intergenic region RE6-9 downstream of Scn5a contacts several sites within this locus including the promoters of Exog, Scn5a, and Scn10a and several REs (Fig. 1a). This is further supported by ChIP-seq data of CCCTC-binding factor (CTCF). CTCF contributes to looping and interactions between promoters end REs, and to TAD boundaries. This demonstrates that RE6-9 is a cardiac-specific enhancer cluster and that its activity is evolutionarily conserved between human and mouse.

RE6-9 strikingly selective controls Scn5a expression. RE6-9 interacts with multiple promoters and is required for the topological organization of the TAD, indicating it may directly or indirectly control expression of multiple genes within the TAD. We assessed the transcript levels of Scn5a and genes in- and outside the TAD in 2-week-old ventricles and brain tissue of RE6-9/+ mice by RT-PCR (Fig. 4a, b). Scn5a was prominently affected in the heart, but not in the brain of RE6-9/+ mice compared to wildtype littermates. In addition, we observed a trend of decreasing Scn10a expression in the heart. The transcript levels of the selected genes surrounding the Scn5a-Scn10a locus were unaffected in the heart and brain (Fig. 4a, b). To validate that the enhancer cluster specifically regulates Scn5a expression in the heart, we performed RNA-sequencing of 2-week-old ventricles of wildtype (n = 3) and RE6-9/+ mice (n = 3). We detected approximately 15,000 transcripts in the ventricles and surprisingly, within the TAD only Scn5a expression was highly significant downregulated (P = 3.75e-27) in RE6-9/+ mice (Fig. 4c-f). We noted that there was a significant decrease in Rnu5g expression. Rnu5g is a 5S small nuclear RNA located about 50 megabases (Mb) away from Scn5a on the same chromosome. These data indicate that RE6-9 is a cardiac-specific enhancer cluster that selectively controls Scn5a.

The two-fold downregulation of Scn5a expression suggests that only the wildtype allele is actively transcribing the gene upon heterozygous deletion of RE6-9. To assess the allele-specific expression of Scn5a we compared the genome of FVB/NJ with the genome of other mouse strains for sequence variations using www.sanger.ac.uk/science/data/mouse-genomes-project. Only SPRET/EiJ contains sufficient sequence variation in Scn5a, Scn10a, and Exog to enable the analysis of allele-specific expression. We generated F1 hybrids of heterozygous RE6-9 transgenic mice (FVB/NJ) crossed to SPRET/EiJ mice. RNA isolated from left ventricular tissue of 8-week-old SPRET;FVB F1 hybrids was used to amplify RT-PCR products containing a genetic variant (SNP) in Scn5a, Scn10a, and Exog followed by Sanger sequencing. In F1 hybrid mice with an unmodified FVB allele, both the SPRET and FVB alleles expressed Scn5a based on the presence of both variants in the RNA (A/T; Fig. 5b, upper panel). In contrast, SPRET;FVBΔRE6-9 F1 hybrid mice only express a transcript from the SPRET-derived allele (A/T; Fig. 5b, upper panel). These data indicate that heterozygous deletion of RE6-9 results in a monoallelic expression of Scn5a, supports the notion that the enhancer cluster is absolutely required for the expression of cardiac Scn5a from the same allele, and argues against inter-chromosomal interaction between the enhancer and Scn5a promoter. In addition, Scn10a and Exog are expressed at the same ratios between the two different alleles in the SPRET; FVB and SPRET;FVBΔRE6-9 F1 hybrids, indicating that any changes in expression of these genes when RE6-9 is deleted are small and not detectable using this assay (Fig. 5b, lower panel).

We further investigated the transcript level of Scn5a in the cardiac compartments of 8-week-old RE6-9/+ mice using quantitative RT-PCR. We observed a 50% reduction in Scn5a expression in different compartments of the heart, except the right atrium, of heterozygous RE6-9 deletion mice (Fig. 5c). A similar downregulation was observed in mice heterozygous for a TALEN-generated 7-bp deletion in coding exon 3 of Scn5a, creating a frameshift with a premature stop codon. This mutation probably results in nonsense mediated decay of the Scn5a mRNA. Heterozygous deletion of RE6-9 and of the 7-bp in Scn5a exon 3 (Scn5aΔ7) resulted in a significant decrease in the Scn5a mRNA level and Na1.5 protein level in the left ventricle (Fig. 5d, e). These data demonstrate that RE6-9 is required for Scn5a expression in multiple parts of the heart. Loss-of-function gene mutations may trigger the induction of genes exhibiting sequence similarity with the mutated gene, a phenomenon called genetic compensation. We measured the expression of sodium
Fig. 3 The three-dimensional organization of the Scn5a-Scn10a locus changes upon deletion of RE6-9. a, b 4C contact profiles of wildtype, n = 3 (WT, blue), RE6-9+/−, n = 3 (orange) and shared signal (gray) with viewpoints set on the Scn5a promoter (a) and the intronic regulatory element RE1 located in Scn10a (b). The intradomain interactions between Scn5a, Scn10a, RE6-9, RE1, and RES are reduced in heterozygous RE6-9 mice. c Model of interaction of the Scn5a-Scn10a locus. Regulatory elements RE1 (green), RES (blue) and RE6-9 (red) with the Scn5a, Scn10a, Exog, and Wdr48 promoters (black arrow).
channel gene family members of Scn5a, and found deregulation of several members in the heart of heterozygous Scn5a<sup>+/Δ</sup> mice (Supplementary Fig. 8). However, expression of these family members was not changed in RE6-9<sup>+/−</sup> mice, showing that reduced expression of Scn5a in RE6-9<sup>+/−</sup> mice by itself does not trigger genetic compensation (Supplementary Fig. 8).

**RE6-9 enhancer integrity is required for Scn5a expression.** To explore the functional integrity of the RE6-9 enhancer cluster depends on individual elements, we established transgenic mouse lines with deletions of elements utilizing CRISPR/Cas9 and TALEN technology (Fig. 5a). Using quantitative RT-PCR we determined Scn5a expression in the ventricles of 8-week-old

Fig. 4 Transcriptome analysis of heterozygous RE6-9<sup>+/−</sup> mice. **a, b** Absolute expression (**a**) and fold change expression (**b**) levels of Scn5a, Scn10a and genes within and outside Scn5a TAD from ventricles (left) and brain (right) from 2-week old RE6-9<sup>+/−</sup> mice compared to wildtype (WT). Bars represent mean ± SEM and P values were calculated using unpaired Student’s t test. WT n = 4; RE6-9<sup>+/−</sup> n = 7. ***P < 0.001. **c** UCSC browser view of the Scn5a-Scn10a locus showing RNA-seq profile of WT and RE6-9<sup>+/−</sup> mice. **d-f** Vulcano plot (**d**), MA plot (**e**) and scatter plot (**f**) of approximately 15,000 transcripts showing only Scn5a is highly significantly (**P = 3.75e-27**) downregulated in RE6-9<sup>+/−</sup> mice compared to WT. P values were corrected for multiple testing using the false discovery rate (FDR) method of Benjamini-Hochberg (**P < 0.05**). Source data are provided as a Source Data file.
heterozygous mutants in which individual or multiple elements of the enhancer cluster were deleted. Heterozygous deletion of RE6-8 (RE6-8+/−) causes a 30% decrease in Scn5a expression, whereas heterozygous deletion of RE9 (RE9+/−) caused a reduction of 50% in Scn5a expression, similar to the reduction in RE6-9+/− mice. In contrast, heterozygous deletion of RE6 (RE6+/−), previously implicated in SCN5A expression and containing a GWAS variant, did not affect Scn5a expression in the ventricles (Fig. 5f).

Next, we assessed the transcript levels of Oxsr1, Exog, Scn10a, and Wdr48 of the enhancer cluster using various established mouse lines (8-week old) with deletions of regulatory elements from the enhancer cluster. Heterozygous deletion of RE6-9 (RE6-9+/−) caused a reduction of 70% in Scn5a expression, whereas RE9+/− and RE6-8+/− mice. Interestingly, we observed a mild decrease in Scn10a and Wdr48 expression after deletion of individual components of the enhancer cluster, whereas Oxsr1 and Exog were unaffected (Fig. 5g). Together, these data indicate that deletion of individual components of the enhancer cluster RE6-9 causes loss of functional integrity of the enhancer cluster RE6-9 is required for its function in the heart and cardiac Scn5a expression. a UCSC browser view of the enhancer cluster RE6-9 and the deletions of regulatory elements from this region by TALEN and CRISPR/Cas9 technology (red rectangles). Included location of the TALENs and CRISPR/Cas9 sgRNAs design and cardiomyocyte-specific H3K27ac ChIP33. b Sequence traces of a genetic variant in Scn5a, Scn10a, and Exog from SPRET, FVB and SPRET;FVB RE6-9 F1 hybrids. c Fold change expression level of Scn5a in different compartments of heart from 8-week old RE6-9+/− mice compared to wildtype (WT). P values were calculated using unpaired Student’s t test. WT n = 4; RE6-9+/− n = 5 (LA, RA, IVS); RE6-9+/− n = 7 (LV, RV). **P < 0.01; ***P < 0.001. d Fold change expression level of Scn5a from left ventricle of 8-week old RE6-9+/− and Scn5a+/Δ7 mice. P values were calculated using unpaired Student’s t test. WT n = 4; Scn5a+/Δ7 n = 4; RE6-9+/− n = 5; *P < 0.05; **P < 0.01. e Fold change expression level of Nav1.5 from left ventricle of 8-week old RE6-9+/− and Scn5a+/Δ7 mice. WT n = 2; Scn5a+/Δ7 n = 2; RE6-9+/− n = 2. f Fold change expression level of Scn5a from various established mouse lines (8-week old) with deletions of regulatory elements from the enhancer cluster. P values were calculated using unpaired Student’s t test. T RE6-9+/− n = 5; C1 RE6-9+/− n = 7; C2 RE6-9+/− n = 5; C3 RE6-9+/− n = 3; C4 RE6-9+/− n = 4; C1 RE9+/− n = 6; C2 RE9+/− n = 5; C3 RE9+/− n = 4; C4 RE9+/− n = 5; T RE6-9+/− n = 5; *P < 0.05; **P < 0.01. g Fold change expression level of Oxsr1, Exog, Scn5a, and Wdr48 of the enhancer cluster RE6-9, RE9, or RE6-8. P values were calculated using unpaired Student’s t test. *P < 0.05; **P < 0.01; ***P < 0.001. Bars represent mean ± SEM.
cluster and subsequently loss of Scn5a expression, and point towards the prominent contribution of RE9 to enhancer cluster activity.

Deletion of enhancer cluster RE6-9 causes conduction slowing. Heterozygous Scn5a mutant mice display a variety of arrhythmias and progressive cardiac conduction disease including prolongation of the PR interval and QRS duration. We recorded in vivo and ex vivo ECG, and performed optimal mapping from 8 to 13-week-old RE6-9+/− mice. ECG recordings indicated heart rates in anesthetized RE6-9+/− mice and wildtype littermates was not different. The ECGs of RE6-9+/− mice revealed a lengthening of the PR interval (wildtype n = 45; RE6-9+/− n = 23) and QRS duration (wildtype n = 44; RE6-9+/− n = 22), whereas other ECG parameters were not altered (Fig. 6a–c). Next, we studied the electrophysiological properties of whole isolated RE6-9+/− hearts in a Langendorff perfusion setup. We observed prolongation of the PR interval in RE6-9+/− hearts (n = 6) compared to wildtype hearts (n = 3) (Fig. 6d–f). Furthermore, episodes of second-degree atrioventricular conduction (AV) block was observed in 3 of 6 RE6-9+/− hearts. Note the autonomic modulation is absent ex vivo, which is of crucial importance for AV conduction in the setting of reduced sodium current or sodium channel dysfunction. We speculate the autonomic balance maintains safety of AV conduction in affected AV nodes of RE6-9+/− mice in vivo.
providing an explanation for the differences in PR prolongation in in vivo and ex vivo ECG recordings.

We measured ventricular conduction velocity of RE6-9+/− mice during central stimulation. Longitudinal conduction velocity was decreased in the left ventricle of RE6-9+/− mice (n = 5) compared to wildtypes (n = 3) (Fig. 6g, h). The latter supports the observed QRS prolongation in vivo ECGs from RE6-9+/− mice (Fig. 6c). The conduction velocity was not lower in the right ventricle (Fig. 6i). Together, these results show that the enhancer cluster is required for electrophysiological homeostasis of the heart.

Although RE6+/− mice did not show altered Scn5a expression, we observed ~30% reduction in Scn5a expression in the left ventricle of homozygous RE6 (RE6−/−) mice (Supplementary Fig. 9). Note, we removed the core of RE6 including the T-box site the homolog of which in human harbors rs6781009. We investigated the functional role of RE6 in myocardial excitability and cardiac conduction by performing in vivo ECG analysis. ECG recordings from 8 to 13-week-old RE6+/− and RE6−/− mice indicated no functional effect on conduction in the adult mouse heart (Supplementary Fig. 9). Moreover, when we intercrossed heterozygous RE6 mutants in a sensitized genetic background carrying an Scn5a loss-of-function allele (Scn5a+/ΔA7), we did not observe an additional or cumulative effect on change in PR interval and QRS duration (Supplementary Fig. 9). These findings indicate that the contribution of RE6 in the regulation of Scn5a is minimal.

### Discussion

In this study, we investigated the physiological role of REs in the SCN5A-SCN10A locus. We identified an evolutionary conserved cardiac enhancer cluster in the locus that controls expression of the SCN5A gene during development and homeostasis. Deletion of the enhancer cluster or one of its components, RE9. Loss-of-function mutations in SCN5A affects conduction and heart function, and cause arrhythmias under particular circumstances. Moreover, homozygous loss of function is embryonic lethal, independent of the 50% current in fish, suggesting SCN5A has additional functions. Therefore, we expected to observe a large number of genes to be indirectly deregulated in adult mutant mouse hearts. Apparently, a 50% reduction in mRNA and protein expression causing conduction slowing does not affect homeostatic gene expression. Recent studies have uncovered a mechanism for genetic compensation, which can occur in response to loss-of-function gene mutations. A key aspect of this mechanism is the requirement of mutant mRNA degradation for the induction of genes with sequence similarity (e.g. paralogous genes). Consistent with these findings, we observed that the Scn5a 7-bp deletion allele, which expresses a mutated mRNA that presumably is degraded, caused deregulation (both induction and reduction) of expression of several paralogous sodium channel-encoding genes, whereas the deleted RE6-9 allele, which is not transcribed, did not. Adaptation has been proposed to provide a mechanism for robustness; alleles that do not transcribe the mutated gene do not show adaptation and may give rise to more severe phenotypes. Our data imply that loss-of-function mutations in SCN5A coding sequences may cause deregulation of other genes, possibly influencing cardiac function, whereas mutations or variants that reduce SCN5A transcription, such as those affecting RE9, will not cause gene deregulation. These data have implications for our understanding of conduction disease-causing mutations affecting SCN5A. It will be interesting to analyze the mutation-induced genetic adaptation in greater detail, and assess how it influences cardiac function and homeostasis.

### RE6-9 is partially redundant during development.

We next analyzed the requirement of the enhancer cluster for development. Homozygous Scn5aΔ7/Δ7 embryos died around E10.5, as was previously observed for Scn5a homozygous loss-of-function mice (Fig. 7a). To assess the regulatory function of RE6-9 during development, we generated compound heterozygous mutants by interbreeding of Scn5aΔ7/Δ7 and RE6-9−/−. Scn5aΔ7/ΔRE6-9− embryos are also lethal at E10.5 and these embryos are phenotypically similar to Scn5aΔ7/Δ7 embryos, revealing that the expression level of Scn5a from the ΔRE6-9 allele is very low during early development (Fig. 7a). RE-9 knockout mutants (RE6-9−/−) survive up to E13.5 (Fig. 7b and Supplementary Table 2). We observed that RE6-9−/− E10.5 embryos still express low levels of Scn5a in the heart (Fig. 7d, f). Furthermore, there was a significant decrease of Scn10a, but no changes in the expression of Exog and Wdr48 (Fig. 7d, f). Inspection of H3K27ac ChIP-seq data at different stages of pre- and postnatal heart development revealed RE6-9 is increasingly enriched for H3K27ac during development (Fig. 7h). These findings suggest that prior to the intermediate embryonic stage (E13.5) other REs (RE1, RE5) act during development to control Scn5a expression in the heart. Thus the enhancer cluster RE6-9 is partially redundant during development. Next, we tested the embryonic lethality in RE6-8 knockout mutants (RE6-8−/−). RE6-8−/− embryos were normal compared to wildtype at E13.5 and we did not observe cardiac defects (Fig. 7c, Supplementary Table 2). Furthermore, the expression of Scn5a, Scn10a, Exog, and Wdr48 were not affected in RE6-8−/− embryos (Fig. 7c, g). These findings suggest that upon deletion of RE6-8 other REs (RE9, RE1, and RE5) operate to regulate Scn5a expression during heart development.

### Genetic variants affecting the enhancer activity of RE6-9.

A number of genetic variants associated with prolonged PR interval and QRS duration map to the RE6-9 cluster (Fig. 1a, b). Because RE9 is a key element within the cluster, we focused on QRS-associated genetic variant rs6810361, positioned in RE9 (Supplementary Fig. 10). RE9 overlaps with a site occupied by T-box transcription factors (Tbx5, Tbx3, Tbx20) as indicated by cardiac ChIP-seq data. We identified a conserved Tbx20 binding consensus sequence in RE9. However, rs6810361 is located at the edge of the T-box binding site (Supplementary Fig. 10). We performed functional analysis on the major allele (i.e., T) and minor allele (i.e., C) of rs6810361 in RE9 in rat heart derived H10 cells. Stimulation with only the cardiac transcription factors Nkx2-5 and Gata4 showed a strong reduction in RE9 enhancer activity for the minor allele in H10 cells. This effect is possibly rescued by Tbx20-mediated regulation as we observed an increase in enhancer activity of RE9 (Supplementary Fig. 10). We observed no difference in activation by Tbx3 and Tbx5 for both alleles (Supplementary Fig. 10). These findings demonstrate that a single nucleotide alteration that is not located in the core element of a T-box transcription factor binding site might affect binding of other cardiac transcription factors, which results in a change in enhancer activity in vitro.
REs are usually located within the non-coding genome and often at large distances from gene promoters. REs can stimulate transcription initiation by interacting with promoters via chromatin looping mechanisms. RE-promoter interactions are largely confined to TADs. RE-promoter interactions have been mapped in various tissue types. However, physical proximity between REs and promoters can be transient, tissue-specific and, importantly, does not predict whether the interaction is functional and influences transcription. Therefore, it remains difficult to predict the target promoter(s) of REs in a particular cell type or condition. We deleted key REs downstream of Scn5a from the mouse genome to define their target genes, tissue-specific functional interactions and mechanism of action. 4C-seq analysis indicated a change in the three-dimensional
Fig. 7 Regulatory function of RE6-9 is partially compensated during development. **a** Analysis of Scn5a+/Δ7 and RE6-9+/− embryos at E10.5. Heterozygous Scn5a and Scn5a mutants develop normally. Compound heterozygous Scn5a+/Δ7/RE6-9 are lethal at E10.5 and are phenotypically similar to Scn5a+/Δ7. **b** RE6-9+/− embryos at E13.5 reveals embryonic lethality. **c** RE6-8/− embryos at E13.5 develop normally and do not show cardiac defects. White scale bars are all 1 mm. **d** Absolute expression level of Exog, Scn5a, Scn10a, and Wdr48 of E10.5 whole heart RE6-9+/− embryos (d) and E13.5 whole heart RE6-8/− embryos (e) compared to wildtype (WT). **f, g** Fold change expression level of Exog, Scn5a, Scn10a, and Wdr48 of E10.5 whole heart RE6-9+/− embryos (f) and E13.5 whole heart RE6-8+/− embryos (g). Bars represent mean ± SEM and P values were calculated using one-way ANOVA with post hoc Bonferroni tests. WT n = 4; RE6-9+/− n = 5; RE6-9+/− n = 5; WT n = 5; RE6-8+/− n = 5; RE6-8+/− n = 7. *P < 0.01, **P < 0.001. **h** UCSC browser view of the enhancer cluster RE6-9 showing H3K27ac ChIP41 at different stage of pre- and postnatal heart. Source data are provided as a Source Data file.
suspension. Chromatin was cross-linked with 2% formaldehyde in PBS with 10% FCS for 10 min at room temperature. Nuclei were isolated and cross-linked DNA was digested with DpnI, followed by proximity ligation. Cross-links were removed and a secondary restriction digestion with Csp61 performed, followed again by proximity ligation. Per viewpoint, 8 PCR reactions were performed on 200 ng of 4C template each, to a total of 1.6 µg. PCR reactions were pooled and purified for next generation sequencing. The 4C-seq PCRs were essentially performed as previously described using standard forward primers (Scn5a_P1+2–CTGCAAGGCGCA-CACGTATGC and Scn5a_enh1—GAGCTCAACAGCTTTAGGATC) as 4C viewpoint combinations with non-reading primers (Scn5a_P1 + 2– CCTCTTAGGGATGCTAG and Scn5a_enh1—ACGCTAGGCGAGCACT-GAC). The Sanger sequencing reads were demultiplexed and subsequently trimmed using FourCseq package python tool "demultiplex.py". Trimmed reads were aligned with bowtie2 (v2.2.5) using standard parameters and quality filter set to 1 (q 1) and processed as described in65. In short, reads are mapped to a restricted mouse reference genome (mm9) consisting of sequences directly flanking the 4C primary restriction enzyme sites (DpnII), termed 4C frag-ends. Non-unique frag-ends are discarded for posterior analysis. The frag-end with highest coverage is removed from the dataset in the normalization process and data are read-depth normalized to 1 million aligned intrachromosomal reads. 4C-seq coverage profile is obtained using "running means", i.e. coverage averages of 21 consecutive 4C frag-ends.

**RNA-sequencing.** Total RNA was isolated from the ventricular tissue of heterozygous RE6-9 mice and wildtype littermates using TRI Reagent (Sigma Aldrich) according to the manufacturer’s protocol (Sigma Aldrich) and further purified using Nucleospin RNA Clean-up Kit with Dnase treatment (Macherey-Nagel). cDNA was prepared with the TrueSeq Stranded Total RNA Library Preparation Kit (Illumina, Part# 15031048 Rev. E). Total RNA (1 µg) was purified and sheared into small fragments followed by cDNA synthesis and ligation of the adapter. The quality and size distribution of the cDNA library templates were validated with Agilent DNA 1000 on 2100 Bioanalyzer (Agilent Technologies). cDNA samples were pooled per lane of paired-end 125 bp sequencing on an Illumina HiSeq 2500 instrument.

**Differential expression analysis.** Reads were mapped to mm10 build of the mouse transcriptome using STAR80. Differential expression analysis was performed using the DESeq2 package based on a negative binomial distribution model87.

**RNA isolation and RT-qPCR.** Total RNA was isolated from cardiac and brain tissue using TRI Reagent (Sigma Aldrich) according to the manufacturer’s protocol (Sigma Aldrich) and treated with Dnase I (Invitrogen). cDNA was reverse transcribed from 500 ng total RNA with oligo-dT primers (125 µM) and the SuperScript II system (Invitrogen). Expression of different genes was assayed with quantitative RT-PCR using the LightCycler 480 Real-Time PCR system (Roche Diagnostics). The amplification protocol consisted of 5 min 95 °C followed by 45 cycles of 95 °C (30 s), 60 °C (15 s) and 72 °C (45 s). Relative gene expression was calculated using the 2^(-Delta Delta Ct) method86.

**Luciferase assays.** RE9 (chr9: 119378700-119379271), major allele, T or minor allele, C, were cloned in pGL2-basic-luciferase construct that contains a minimal promoter. Luciferase constructs were co-transfected with pcDNA3.1 constructs expressing Gata4, Nkx2-5, Tbx20. Constructs were transfected in H10 cells with the PEF transfection reagent (Burlingham). Standard transfections used 1.0 µg of reporter construct (or control reporter construct) co-transfected with 3 ng pBG-TK Renilla vector (Promega) as normalization control. Luciferase measurements were performed using a Promega Turner Biosystems Modulomo Multimode Reader Luminometer.

**Statistical analysis.** Results are expressed as mean ± SEM. Two group mean differences were evaluated using standard two tailed t test and more than two groups were compared using one way ANOVA followed by post hoc Bonferroni test. For luciferase assays statistics was validated using a two-way ANOVA test. Statistical significance was considered for P < 0.05.

**Data availability.** RNA-seq data and 4C-seq data were deposited to the Gene Expression Omnibus under accession number GSE129067. All other relevant data supporting the key findings of this study is available within the article and its Supplementary Information files or from the corresponding author upon reasonable request. Detailed information on statis test the 4C-seq data per replicate is available in Supplementary Table 5. Data underlying Figs. 4a, b, d–f, 5c–g, 6b, c, e, f, h, i; 7d–g and Supplementary Figures 8a, b, 9a–d; 10c in this study is provided as a Source Data file. A reporting summary for this Article is available as a Supplementary Information file.

**Mouse transgenic enhancer assay.** The regulatory region RE6-9 (chr9: 11937827-11938949) was PCR amplified from BAC RP23-1981L19 using the primers forward—GTACCTCCAAACAAGTGGAGAAGAC and reverse—TCTGCAACCTCTATAGGGAAACA and was cloned into the TOPO vector (Invitrogen). Insert RE9 was modified by restriction enzyme digestion analyses and sequencing, and recombined into a Hsp68-LacZ-Gateway reporter vector using Gateway LR Clonase II Enzyme Mix kit (Invitrogen)88. Generation of transgenic embryonic stem cells was performed by pronuclear injection with protocols approved by Cyagen Biosciences Inc., Santa Clara, CA.

In vivo ECG recording. Animals were anaesthetized with 1.5% isoflurane. Electrodes were placed at the right (R) and left (L) armpit and the left groin (F). A reference electrode was placed at the right groin. ECGs were recorded (Biosemi, Amsterdam, the Netherlands; sampling rate 2048 Hz, filtering DC 400 kHz (5 dB)) for a period of 2 min. From these leads, a standard three-lead ECG was calculated as follows: I = L – R, II = F – R, III = F – L, aVR = R – (L + F)/2, aVL = L – (R – F)/2, and aVF = F – (L + R)/289.

**Langendorff experiments.** Mice were stunned by inhalation of CO₂ and killed by cervical dislocation, after which the heart was excised, cannulated, mounted on a Langendorff perfusion setup, and perfused at 37 °C with Tyrode’s solution (122 mmol/L NaCl, 4.7 mmol/L KCl, 1.45 mmol/L CaCl₂, 0.6 mmol/L MgCl₂, 27 mmol/L NaHCO₃, 0.4 mmol/L NaH₂PO₄, and 11 mmol/L glucose (pH maintained at 7.4 by equilibration with a mixture of 95% O₂ and 5% CO₂)). Ex vivo ECGs were recorded (Biosemi, Amsterdam, the Netherlands; sampling rate 2048 Hz, filtering DC 400 kHz (3 dB)) and analyzed using LabChart Pro (by Mitchells formular). Activation patterns were measured during sinus rhythm and atrioventricular and atrial pacing at a basic cycle length of 120 ms (twice the diastolic stimulation threshold). The effective refractory period of the AV node was determined by atrial pacing and reducing the coupling interval of a premature stimulus (after 8 stimuli at basic cycle length of 120 ms) in steps of 5 ms until activation of the ventricle failed33.

**Sanger sequencing.** DNA sequencing was performed with BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The BigDye Cycle Sequencing reaction utilized the following cycling parameters: initial denaturation at 96 °C (10 s), denaturation at 96 °C (30 s), with an intervening 40 cycles of prime annealing at 55 °C (15 s), elongation 60 °C (4 min). Sequencing is outsourced to the AMC Sequence Facility. Results are retrieved via LIMS from the AMC Sequence Facility and analyzed with CodonCode Aligner (CodonCode Corporation).

**Western blot.** Total membrane fraction was isolated from left ventricular tissue using ice-cold lysis buffer (5 mmol/L Tris·HCl pH 7.5, 2 mmol/L EDTA) supplemented with a protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics) and then centrifugated at 380g at 4 °C to remove cell debris. Whole cell lysate was centrifuged at 18620g for 30 min. The pellet containing the total membrane fraction was resuspended resuspension buffer (55 mmol/L Tris·HCl pH 7.5, 5 mmol/L EDTA, 12.5 mmol/L MgCl₂) supplemented with a protease inhibitor cocktail tablet. Protein concentrations were determined using the Protein Assay Reagent BCA kit (Thermo Fisher Scientific). Protein samples (50 µl) of the total membrane fractions were heated to 95 °C for 5 min in Laemml buffer (5x Laemmle buffer: 300 mmol/L Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 25% 2-mercaptoethanol, 0.02% bromophenol). Then protein samples were loaded on 8% SDS-PAGE gel followed by standard Western blotting (trans-blot semi dry transfer cell machine; Biorad 470-3940, Immobilon-PSQ transfer membrane; Millipore). Blots were incubated with Nav1.5 (1:300, Sigma-Aldrich, 50819) or CalcineX (1:2000, Millipore, WVR208880) overnight at 4 °C. A HRP conjugated secondary antibody (1:2500, GE Healthcare Life Sciences) was applied and incubated for 2h at room temperature. Immuno-reactive bands was visualized using chemo-luminescent ECL-PLUS Western blot reagent (Amer sham Pharmacia Biotech) and luminescence recording was performed with the ImageQuant LAS 4000 Bio-molecular image analyzer. Protein concentration was quantified using ImageJ. Source data of uncropped blots are provided as a Source Data file.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Received: 21 November 2018; Accepted: 3 October 2019; Published online: 30 October 2019

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Acknowledgements
This study was supported by CVON HUSTCARE, Charles River grant and by a Leducq Foundation grant (14CVD01).

Author contributions
J.C.K.M. performed the 4C experiment, RNA-sequencing, in vivo mouse experiments, analyzed and interpreted the data. M.B. performed the in vitro H10 cells experiment and in vivo mouse experiments, analyzed and interpreted the data. C.R.E.H. performed the 4C experiment, analyzed and interpreted the data. C.J. performed the electrophysiological experiments, analyzed and interpreted the data. V.W. performed the in vivo mouse experiments, analyzed and interpreted the data. V.B. contributed to data interpretation. B.J.B. performed the electrophysiological experiments, analyzed and interpreted the data. V.M.C. conceived the project, designed the experiments, interpreted the data and wrote the manuscript.

Competing Interests
W.d.L. is founder and shareholder of Cergentis.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-12856-5.

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Peer review information Nature Communications thanks Peter Backx and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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