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Abstract

The cardiac conduction system (CCS) consists of distinctive components that initiate and conduct the electrical impulse required for the coordinated contraction of the cardiac chambers. The development of the CCS involves complex regulatory networks of transcription factors that act in stage, tissue and dose-dependent manners. As disrupted function or expression of these factors may lead to disorders in the development or function of components of the CCS associated with heart failure and sudden death, it is crucial to understand the molecular and cellular mechanisms underlying their complex regulation. Here, we discuss the regulation of genes driving CCS-specific gene expression and demonstrate the complexity of the mechanisms governing their regulatory networks. The three-dimensional conformation of chromatin has recently been recognized as an important regulatory layer, shaping the genome in regulatory domains and physically wiring gene promoters to their regulatory sequences. Knowledge of the mechanisms by which distal-acting regulatory sequences exert their function to drive tissue-specific gene expression and understanding how the three-dimensional chromatin landscape is involved in this regulation will increase our understanding of how disease-associated genomic variation affects the function of such sequences.

Keywords

Heart development • Conduction system • Sinus node • Atrioventricular node • Transcriptional regulation • Functional genomics • Patterning • Pacemaker

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38.1 Introduction

The cardiac conduction system (CCS) initiates and propagates the electrical impulse that is required for the rhythmic and synchronized contraction of the heart. The impulse initiates in the sinoatrial node (SAN) and is rapidly propagated through the atria, thereby activating the contraction of the atrial myocardium. The impulse is then propagated through the atrioventricular node (AVN), the only electrical connection between the atria and ventricles. The AVN delays conduction of the impulse, allowing for the atrial contraction and ventricular filling to complete before the ventricles contract. Further propagation of the impulse to the fast-conducting atrioventricular bundle (AVB), bundle branches (BBs) and Purkinje network causes the depolarization of the ventricular myocardium, leading to ventricular contraction. The development of the CCS is regulated by transcription factors that act in strictly stage, tissue and dose-dependent manners [1, 2]. A disruption in the function or expression of these factors could lead to disorders in the development or function of the CCS that can lead to lethal arrhythmias and heart failure. Knowledge of the mechanisms underlying regulation of genes involved in CCS development is therefore crucial.

38.2 Genetic Pathways Controlling SAN and AVC Development

The heart is the first organ to form during embryonic development and starts as a primitive, linear tube with the inflow region at the caudal side and the outflow region at the cranial side. The slow conductive properties of the embryonic muscle cells within the primitive tube at this stage and dominant pacemaker activity at the caudal end cause a slow peristaltic pattern of contraction along the tube to propagate the blood. At this stage, the entire sinus venosus acts as pacemaker, characterized by the expression of the pacemaker channel *Hcn4*, a member of the family of channels responsible for the hyperpolarization-activated current *if* that is crucial for the pacemaker potential [3, 4]. The heart tube elongates by the addition of rapidly proliferating progenitor cells that differentiate to cardiac muscle. This implies that cells added to the inflow tract will acquire dominant pacemaker activity. With further development, specific regions in the heart tube start to divide rapidly and activate a working myocardial gene program, resulting in the ballooning of the primitive atrium and ventricle. Concomitant with the ballooning of the primitive atria is the formation of the sinus venosus including the SAN. Dominant pacemaker activity will gradually be confined to the SAN at the junction of the sinus venosus and atrium.

The transcriptional activator *Tbx5* is required for the sinus venosus expression of *Shox2* [5], a homeobox transcription factor necessary for SAN formation and function [6, 7]. *Shox2* represses cardiac homeobox transcription factor *Nkx2-5* [6]. In the chambers, *Nkx2-5* activates chamber-specific genes including *Nppa* and high-conductance gap junction subunit-encoding genes *Gja5* (Cx40) and *Gjal* (Cx43), whereas it represses SAN/CCS-specific genes *Hcn4* and T-box transcription

factor *Tbx3* [8]. *Tbx3* is required for the formation of the SAN by directly repressing atrial myocardial genes *Gja5*, *Gjal* and *Nppa* to prevent atrialization of the SAN and indirectly activating *Hcn4* and other SAN genes [9, 10].

During ballooning of the primitive cardiac chambers, the region in between the atria and ventricles does not proliferate and forms a constriction, the atrioventricular canal (AVC). *Bmp2* expression in the AVC activates the expression of *Tbx3* and *Tbx2* [11]. Together with *Msx2*, these T-box factors repress the working myocardial gene program in the AVC and AVC-derived AVN [12, 13] and stimulate the pacemaker gene program and the program required for the formation of the AV cushions. Within the AVC, *Tbx2* and *Tbx3* interact with *Nkx2-5* to repress genes that are activated by *Nkx2-5* and *Tbx5* in the working myocardium of the atria and ventricles [14–16]. *Tbx2* and *Tbx3* thus suppress working myocardial differentiation of the AVC, thereby causing the retention of the primitive phenotype of slow conduction and low rates of proliferation, providing a primitive morphological and functional constriction in between the atrial and ventricular chambers. Other factors that regulate the formation of the AVC and its border with the chamber myocardium include *Wnts*, acting upstream of *Bmp2*; *Hey1* and *Hey2*, Notch target genes expressed in the chambers that suppress *Tbx2* [17]; *Tbx20*, which represses BMP-mediated activation of *Tbx2* in the chambers [18]; and *Gata4/6*, which act in complex with *Smads* and histone acetyltransferases (HATs) to activate AVC-specific enhancers in the AVC and with histone deacetylases (HDACs) and *Hey1/2* to suppress these enhancers in the chambers [19]. The resulting pattern of conduction—fast in the atria, slow in the AVC and fast in the ventricles—results in the alternating contraction pattern of the chambers and an ECG that resembles the adult ECG (Fig. 38.1a).

38.3 Transcriptional Regulation of CCS Genes

Although the expression patterns and functions of genes involved in the development of the cardiac conduction system are relatively well studied, little is known about the molecular mechanisms underlying their regulation of expression. Tissue-specific gene expression often involves long-range regulatory elements, such as enhancers, which dictate the strictly time-, tissue- and dosage-dependent expression of their target genes. The identification and function of such enhancers is therefore highly relevant to fully understand the complex regulatory networks in CCS formation. However, to date only few studies have been carried out investigating in depth the regulation of genes driving CCS development.

The T-box transcription factor *Tbx5* plays indispensable roles in the early patterning of the heart and CCS and is involved in limb development [15, 20, 21]. Mutations in *TBX5* are associated with Holt-Oram syndrome, a developmental disorder characterized by hand-heart defects [22, 23]. Using modified bacterial artificial chromosomes (BACs), the regulatory landscape of the *TBX5* locus was determined, and within this landscape, multiple cardiac-specific enhancers were identified by utilizing multiple genome-wide ChIP-seq datasets and evolutionary

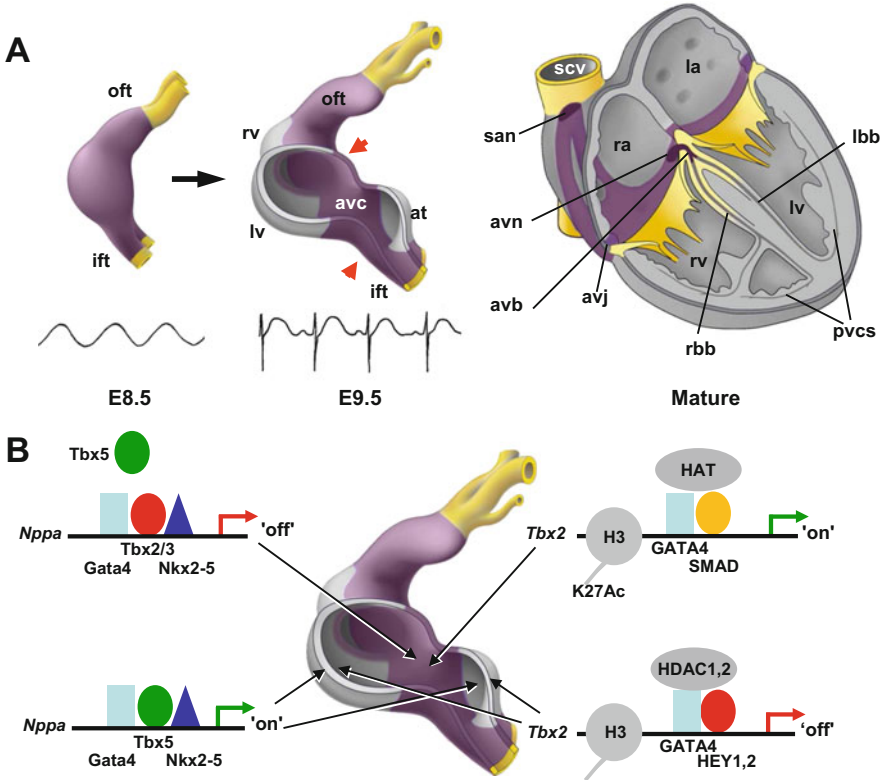


Fig. 38.1 Schematic overview of cardiac development. (a) The early heart tube has a primitive phenotype of slow conduction, represented by a sinusoidal ECG. With further development, regions at the outer curvatures of the primary heart tube expand and obtain a working myocardium phenotype of fast conduction (*grey*). The sinus venosus (*sv*), AVC, outflow tract (*oft*) and inner curvatures retain their primitive pacemaker-like phenotype and slow conductivity (*purple*). A more mature ECG can be derived from these hearts. Eventually, these non-chamber myocardial regions will give rise to the mature conduction system components. (b) The transcriptional repressors *Tbx2* and *Tbx3* compete with the transcriptional activator *Tbx5* to regulate their target genes. In the AVC, expression of chamber myocardium genes like *Nppa* is actively repressed by *Tbx2* and *Tbx3*, whereas in the developing chambers *Tbx5* activates these genes. Transcriptional activation in the AVC is regulated by GATA binding site-dependent histone modifications which render the chromatin more (e.g. HATs) or less (e.g. HDACs) accessible for transcription factors to bind their target regulatory sequences, resulting in activation or repression of gene expression

conservation. These enhancers were shown to recapitulate part of the *TBX5* expression pattern in the heart, but interestingly, none of these fragments drove reporter expression in the limbs, suggesting that the *cis*-regulation of *TBX5* in the heart and limbs is compartmentalized [24]. Such knowledge can highly improve the understanding of the mechanisms underlying the development of congenital heart diseases by decoupling the heart and hand phenotypes seen with Holt-Oram syndrome, thereby presenting more compartmentalized phenotypes compared to disorders caused by protein-coding mutations.

Another example of how enhancer-mediated gene expression is involved in the tight regulation of the CCS is presented by the transcriptional repressor *Id2*. This factor was identified by serial analysis of gene expression (SAGE) as having CCS-specific expression. *Id2* is expressed throughout development in the AVB and BB and in non-CCS compartments such as the AV endocardial cushions and valves. The requirement of *Id2* for ventricular CCS structure and function was demonstrated as *Id2*-deficient mice exhibit structural and functional conduction system abnormalities, including left bundle branch block. *Id2* is cooperatively regulated by *Nkx2-5* and *Tbx5* in the developing ventricular conduction system by binding of both *Tbx5* and *Nkx2-5* to a 1052 bp fragment of the *Id2* promoter. Mutation of the *Tbx5* binding site within this promoter region completely abolished CCS expression whereas extracardiac expression was unaltered, illustrating the specificity of this transcriptional mechanism in the coordinated development of the ventricular conduction system [20].

The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. Using a transgenic BAC approach, it was shown that the regulatory regions sufficient to recapitulate the endogenous *Hcn4* expression pattern in the SAN, AVN, His bundle, bundle branches and left ventricular Purkinje fibres reside within the region covered by one bacterial artificial chromosome (BAC) of 200 kbp [25]. Using transgenic mouse assays, multiple evolutionary conserved cis-acting regulatory sequences were identified to drive *Hcn4* expression in the AV conduction system. One of these regions drives reporter expression specifically in the non-chamber myocardium in a *Mef2c*-dependent manner. Furthermore, depletion of histone deacetylases resulted in ectopic expression of reporter activity in chamber myocardium, revealing a role for histone modifications in *Mef2c*-regulated enhancer-mediated expression of *Hcn4* in components of the CCS [26].

More recently, Contactin-2 (*Cntn2*), a cell adhesion molecule critical for neuronal patterning and ion channel clustering, was described as a marker for the ventricular conduction system, with expression in the AVB, BBs and Purkinje fibres. Using a GFP-modified BAC, the boundaries of the regulatory domain involved in the control of *Cntn2* expression were identified, since reporter activity of the modified BAC completely recapitulates endogenous *Cntn2* expression [27]. Such knowledge facilitates in the identification of single, individual regulatory elements driving CCS development and will greatly add to our understanding of how genes involved in the complex development of CCS components are regulated.

Enhancer function is regulated by modifications of specific histone tails that mark active or poised enhancers. Active enhancers are associated with an open, accessible chromatin state, whereas poised enhancers are associated with dense, closed chromatin. Histone modifiers such as histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone acetyltransferases (HATs) therefore regulate the accessibility of long-range regulatory sequences, allowing for the binding by cell type-specific transcription factors to activate transcription in a tissue-dependent manner. Specification of the AVC is regulated by *Gata4*, which activates AVC enhancers in synergy with *Bmp2*/*Smad* signaling to recruit HATs

such as p300 [19, 28]. This leads to H3K27 acetylation, a marker of active enhancers. In contrast, in chamber myocardium, Gata4 cooperates with HDACs and chamber-specific genes Hey1 and Hey2, leading to H3K27 deacetylation and repression (Fig. 38.1b) [19].

38.4 Common Genomic Variants Influence CCS Function

The importance of the strict regulation of the spatial and temporal expression of CCS genes is illustrated by findings from recent genome-wide association studies, which revealed common genomic variation to be associated with conduction parameters like PR interval and QRS duration. Such variation was identified in non-coding regions flanking genes encoding ion channels like *SCN5A/10A*, *KCNQ1* and *KCNH2* and cardiac transcription factors like *NKX2-5*, *MEIS1* and *TBX3/5*, indicating they might affect the function of enhancers controlling the precise regulation of these genes [29–31]. Tbx5 is broadly expressed and acts as transcriptional activator, inducing transcription of genes involved in cardiac differentiation [15, 20]. The activity domain of Tbx3 is much more restricted and confined to the developing and mature CCS, where it acts as a transcriptional repressor, thereby imposing the pacemaker phenotype on cells within its expression domain [10, 32]. Tbx3 and Tbx5 both recognize the same regulatory sequences [33], suggesting that these factors compete for binding and implicating a fine balance between activation and repression of CS genes by these factors. The precise regulation of transcription and activity of both factors is therefore crucial for proper CCS patterning, and minor changes in regulatory elements controlling the regulation of expression of these factors could thus potentially have large consequences for CCS function and development. Knowledge of the mechanisms by which such developmental genes are regulated to exert their spatio-temporal transcriptional activity is therefore crucial in the understanding of how variation identified by GWAS influences development.

38.5 3D Architecture Regulates Transcription

Physical enhancer-promoter contacts are a requirement for enhancer-mediated cell type-specific gene expression, and as such, the three-dimensional topology of chromatin plays an indispensable role in gene regulation by physically wiring long-range regulatory sequences with their target promoters [34]. Several protein complexes, including CTCF, cohesin and mediator, have been proposed to be involved in the organization of these contacts [35]. Furthermore, recent data suggest that such genomic structural organizers not only mediate single enhancer-promoter contacts but also mediate the organization of the genome in relatively cell-type invariant topologically associated domains (TADs) within which sequences particularly contact each other. Genes located within the same TAD exhibit greater expression correlation than genes located in distinct ones,

suggesting that such domains may act as a backbone for tissue-specific regulatory contacts [36]. The recent emergence of techniques aimed at capturing the 3D conformation of genomic loci [37] therefore provides valuable tools to elucidate regulatory mechanisms on the chromatin level.

38.6 Regulation of *Tbx3* by a Large Regulatory Domain

The evolutionary conserved *Tbx3/5* genomic locus is one of the few genomic loci of which the 3D architecture has been studied and reveals an example of the complexity of gene regulation on the level of chromatin topology. *Tbx3* and *Tbx5* form an evolutionary conserved gene cluster derived from a primordial T-box gene [38] and, as mentioned above, play crucial roles in the formation and function of the cardiac conduction system. Using circular chromosome conformation capture sequencing (4C-seq), which captures all the genomic regions in close proximity to a chosen point of view [39], the 3D architecture of the *Tbx3/5* locus was probed, and genomic regions contacting *Tbx3* or *Tbx5* were identified in different tissues (Fig. 38.2a). Interestingly, these data revealed that the regulatory landscape is in a preformed conformation that is similar in embryonic heart, brain and limb. Rather than the de novo formation of enhancer-promoter loops upon binding by cell-type relevant transcription factors to initiate transcription, the locus is in a fixed, permissive structure in which enhancer-promoter loops are pre-existing [40]. Such a permissive structure has previously been described for different loci, including the *Hox* and *Shh* gene loci. Long-range enhancer-promoter contacts in these loci were shown to be irrespective of cell type, revealing a preformed topology [36, 41]. The permissive, preformed nature of these loci was exemplified by the fact that even in the absence of a distal-acting *Shh* enhancer, contacts between the *Shh* promoter and the enhancer region still occur [41]. The benefit of such preformed regulatory landscapes is believed to lie in the ease by which tissue-specific transcription factors can utilize preformed contacts to target the gene of interest, involving only slight variations in internal contacts within an otherwise rigid and conserved structure. In agreement with this, small differences in contact profiles for the different tissue types were observed in the *Tbx3/5* locus despite the fact that the domain is largely preformed, most probably caused by cell type-specific transcription factor mediated enhancer activation. Among the multiple sites contacting *Tbx3* in the gene desert upstream of the gene, two evolutionary conserved enhancers have been identified that also contact each other. They are bound by cardiac-specific transcription factors *Nkx2-5*, *Gata4*, *Tbx5* and *Tbx3* and were shown to respond to a BMP-mediated signalling pathway to drive atrioventricular conduction system expression of *Tbx3* [40].

As mentioned before, *Tbx3* and *Tbx5* are expressed in overlapping patterns and have overlapping functions in CCS development. It could therefore be expected that both genes share common regulatory mechanisms on a genomic level. Studies on the transcriptional regulation of other clustered developmental genes, like the *Irx* and *Hox* clusters, revealed that regulatory sequences are not uniquely associated

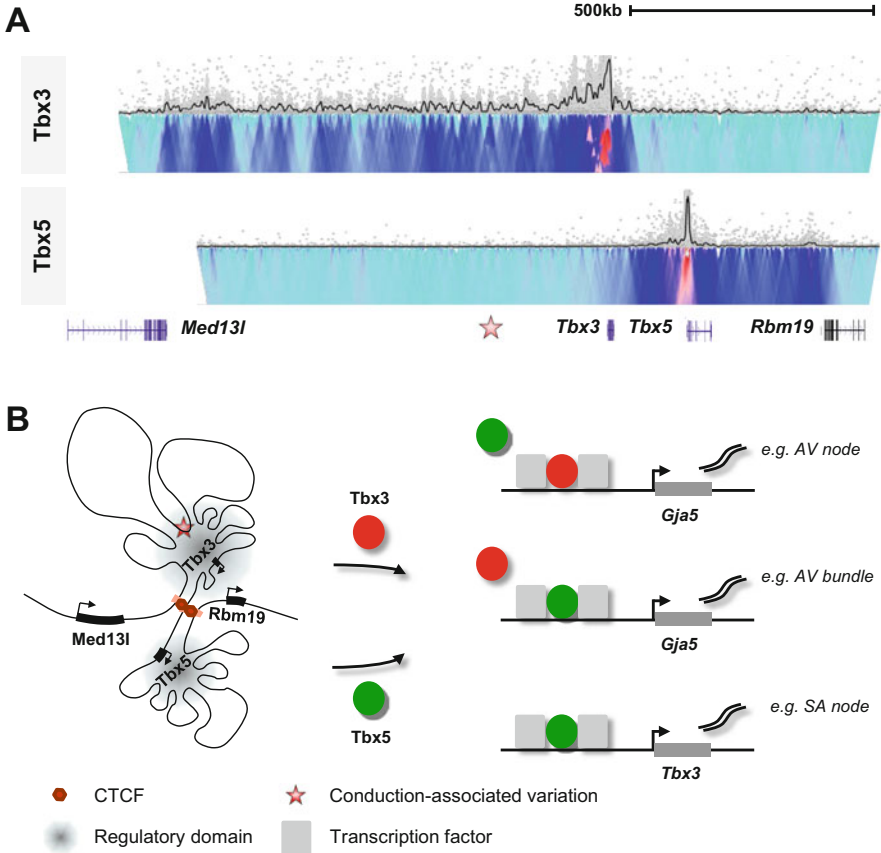


Fig. 38.2 The regulatory domains of *Tbx3* and *Tbx5* are physically separated. (a) Contact profiles of the *Tbx3* and *Tbx5* loci as determined by circular chromosome conformation capture (4C) reveal the genomic regions that physically contact *Tbx3* (upper track) or *Tbx5* (lower track) in mouse embryonic heart cells. Red and dark blue depict a high contact frequency, whereas light blue and grey depict a low contact frequency. The contact profiles of *Tbx3* and *Tbx5* hardly overlap, indicating that both genes do not share regulatory sequences and suggesting that common variants in humans upstream of *TBX3* as identified by GWAS (star) can be exclusively assigned to *TBX3* [40]. (b) Model of the 3D conformation of the *Tbx3/Tbx5* locus. The regulatory domains of *Tbx3* and *Tbx5* are physically separated; however, on the protein level, both genes recognize the same binding sequences and compete with each other to activate or repress their target genes, e.g. *Gja5* (Cx40). Despite the strict regulation on the chromatin level, *Tbx5* also directly regulates *Tbx3* by binding target sequences to activate transcription in, for example, the SAN

with single promoters, but rather are shared by multiple genes within the cluster. Such interplay between multiple enhancers coordinates the strict regulation of their expression patterns, and it has been proposed that such extensive enhancer sharing explains the conservation of the genomic organization throughout evolution [42, 43]. Interestingly, however, *Tbx3* and its flanking gene desert form a loop that is physically separated from that of the neighbouring *Tbx5* loop (Fig. 38.2b).

Genomic regions within the *Tbx3* loop solely contact *Tbx3* but not *Tbx5* and vice versa, indicating enhancer sharing between these evolutionary conserved clusters is unlikely to occur [40]. The strict separation of the regulatory landscapes of *Tbx3* and *Tbx5* is not only cell type-independent, but also evolutionary conserved between mouse and human. Recent Hi-C data in human fibroblasts [36], revealing genome-wide contact profiles, reveal a similar separation of the *TBX3* and *TBX5* regulatory domains with hardly any overlap of the contact profiles. This organization of the *Tbx3* locus in a ~1 Mb-scale self-regulatory domain corresponds well to the previously mentioned TADs. These domains are separated by boundary regions enriched for insulator binding protein CTCF, housekeeping genes, transfer RNA and short interspersed elements, hampering interactions of sequences within one TAD with regions exceeding the domain boundaries. Regions located within the regulatory domain of *Tbx3* are thus suggested to exclusively contact *Tbx3* and not *Tbx5* and vice versa.

38.7 Assigning Function to Genomic Variation

Understanding the 3D architecture of a genomic locus not only provides insight into the tight relationship between chromatin topology and the complex regulation of developmental gene expression, it could also provide valuable clues in the understanding of the role of functional variation as identified by genome-wide association studies on gene expression. Common genomic variation in the non-coding region upstream of *TBX3* and *TBX5* was found to influence PR interval and QRS duration in humans [29–31]. The fact that the regulatory domains of *TBX3* and *TBX5* are strictly separated indicates that the variation found in one of the domains can be exclusively assigned to its respective gene, facilitating our understanding of the functional effect of disease-associated variation.

A similar example of how knowledge on the chromatin conformation can increase our understanding of function of common variants is illustrated by studies on the *SCN5A/SCN10A* locus. *SCN5A* and *SCN10A* encode sodium channels important for conduction. GWAS implicated an intronic region in *SCN10A* as a major risk region for prolonged QRS duration [30]. The role of *SCN10A* in cardiac conduction however was not previously described, whereas mutations in the adjacent *SCN5A* are well established to cause several arrhythmogenic disorders, including Brugada and Long QT syndrome [44, 45]. It is therefore possible that the variation identified within the intron of *SCN10A* impacts the expression of *SCN5A*, rather than or in addition to that of *SCN10A*. Indeed, probing the 3D architecture with the promoters of both *SCN5A* and *SCN10A* and the site of the variation in the intron as point of view using 4C-seq revealed that this variant region contacts the *SCN5A* promoter and a strong enhancer downstream of *SCN5A*, suggesting that it might act as enhancer regulating the expression of *SCN5A* in the heart. Transgenic reporter assays revealed that indeed this enhancer is essential for cardiac *Scn5a* expression. In humans, the SNP located within the enhancer that correlates with slowed conduction is associated with lower *SCN5A* expression

[46]. Taken together, these results provide another example of how our understanding of the 3D architecture of a genomic locus harbouring functional variation can facilitate in the assignment of function to such variations, improving our understanding of the effect of disease-associated variation.

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