The formation and function of the cardiac conduction system

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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. CCS development involves complex regulatory networks that act in stage-, tissue- and dose-dependent manners, and recent findings indicate that the activity of these networks is sensitive to common genetic variants associated with cardiac arrhythmias. Here, we review how these findings have provided novel insights into the regulatory mechanisms and transcriptional networks underlying CCS formation and function.

KEY WORDS: Cardiac conduction system, Cardiac development, Gene regulation, Transcriptional network

Introduction

Heart function starts early during embryogenesis and is crucial to supply the embryo with nutrients and oxygen. From the beginning of its formation, the heart itself generates and propagates the electrical impulse that is required to initiate coordinated contractions to efficiently pump blood throughout the body. In the developed heart, these functions are performed by the cardiac conduction system (CCS), which is made up of various components that each carry out a particular task (Fig. 1). For example, specialized pacemaker myocytes in the sinoatrial node (SAN), which is located at the junction of the right atrium and the superior caval vein, generate the impulse. The impulse is then rapidly propagated through the atrial myocardium and reaches the atrioventricular node (AVN), where it is slowed down. This delay allows the atria to contract and allows the ventricles to fill before the ventricles themselves are activated and contract. The atrial and ventricular myocardial tissues are electrically isolated from one another by a plane of connective tissue formed by the annulus fibrosus and central fibrous body. The only electrical passage from atrial to ventricular myocardium is formed by the fast-conducting atrioventricular bundle (AVB), or His bundle, which is connected to the AVN and runs through the crest of the ventricular septum. It conducts the impulse to the left and right bundle branches (BBs) and the Purkinje fibre network, which activates the ventricular myocardium (Fig. 1). Together, these fast-conducting structures (the AVB/His bundle, the left and right BBs, and the Purkinje fibre network) are referred to as the ventricular conduction system (VCS).

Congenital anomalies or diseases resulting in impaired development or function of CCS components can lead to severe arrhythmias that require therapeutic (e.g. ion channel blockers) or surgical (e.g. ablation, electronic pacemaker implantation) intervention (Wolf and Berul, 2006). Although progress has been made in understanding and analysing (e.g. via electrocardiograms; see Box 1) the electrophysiological properties of CCS structures (Mangoni and Nargeot, 2008; Christoffels et al., 2010; Munshi, 2012), the molecular mechanisms controlling CCS development are still insufficiently understood. However, the recent emergence of novel technologies for investigating tissue-specific transcription regulatory mechanisms and genetic networks at genome-wide levels has provided new avenues into understanding CCS development and homeostasis. In this Review, we highlight recent insights into the transcriptional mechanisms underlying CCS development and homeostasis, and discuss how these findings point to a transcriptional network for the CCS that is sensitive to genetic variation and disturbances.

Early development of the CCS: setting up the building plan of the heart

Early in development, during folding of the embryo, fusion of the two heart-forming regions in the lateral plate mesoderm results in the formation of the primitive heart tube (Fig. 2A). At this time, the myocardial cells of the primitive tube are automatic (i.e. they spontaneously depolarize), slowly conduct the electrical impulse, and have underdeveloped sarcomeres and sarcoplasmic reticulum, leading to poor contraction properties. Dominant automaticity (i.e. pacemaker activity) is located caudally in the venous part of the heart tube, in a region referred to as the inflow tract (IFT). In chick embryos, the first occurrence of pacemaker activity is observed as early as the 7-8 somite stage, even before the recording of the first heartbeat (van Mierop, 1967; Kamino et al., 1981). The embryonic heart tube forms the definitive left ventricle and atrioventricular canal (AVC). The tube elongates by addition of myocytes derived from second heart field (SHF) progenitor cells to both poles of the heart. These will form the definitive right ventricle, the outflow tract (OFT), the atria and the sinus venosus (SV) (Kelly et al., 2014; Meilhac et al., 2014). Throughout heart tube elongation, dominant pacemaker activity is present in the inflow region, implying that cells added to the venous pole of the heart rapidly acquire a pacemaker phenotype that dominates over that of the existing myocytes (de Haan, 1965). As a result, unidirectional waves of contraction travel across the expanding tube towards the arterial pole, reflected by a sinusoidal electrocardiogram (ECG) (Fig. 2A).

During the subsequent looping of the heart tube, regions at the outer curvatures of the tube proliferate extensively and expand to form the future atrial and ventricular chambers (Fig. 2B). The chamber myocardial gene programme activates the expression of, amongst many others, genes encoding subunits of the high conductance gap junctions Cx40 and Cx43 (Gja5 and Gja1, respectively) and the cardiac sodium channel Scn5a (also known as Nav1.5), all of which are essential for conduction. Furthermore, genes encoding sarcomere components and factors that regulate mitochondrial activity are upregulated, directing the cardiomyocytes in the developing chambers towards a working myocardial phenotype of fast conduction and high contractility.

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The resulting configuration of the embryonic heart – with dominant pacemaker activity at the inflow, rapid activation of and impulse propagation through the atria, retention of the slow-conducting phenotype in the AVC, and rapid activation of the ventricle(s) – gives rise to a more mature-like ECG (Fig. 2B). Furthermore, the slow relaxation characteristics of the myocytes in the cushion-filled AVC prevent blood from flowing back into the atria during ventricular activation and contraction, a role later adopted by the mature atrioventricular (AV) valves. The basic activation pattern of the heart is thus already established at an early developmental stage, by embryonic day (E) 9.5 in mice and by Hamburger Hamilton (HH) stage 13 in the chick (van Mierop, 1967; Paff et al., 1968), even though the components of the mature CCS are not morphologically recognizable at this stage yet (Fig. 2B).

The origin and development of the SAN

The SAN develops within the SV myocardium and can be recognized morphologically from E11.5 onwards in mouse, in the right sinus horn at the junction with the atrium (Virágh and Challice, 1980). The initially formed heart tube expresses the core cardiac homeobox transcription factor Nkx2-5. Between E9-9.5 and E11.5-12.5, Nkx2-5 SV myocardium is added to the venous pole by the differentiation of Tbx18\(^+/\)Nkx2-5\(^{-}\) progenitors (Christoffels et al., 2006; Mommersteeg et al., 2007; Wiese et al., 2009). The inflow tract cells of the E9.5 heart tube develop into atrial cells. Hcn4, initially expressed in the first-formed myocytes, is immediately activated in this Tbx18\(^+/\)/Nkx2-5\(^{-}\) domain and is downregulated in the Nkx2-5\(^{+}\) myocardium, thereby effectively shifting its expression domain to the newly added SV and the primary pacemaker domain (Garcia-Frigola et al., 2003; Mommersteeg et al., 2007; Liang et al., 2013; Später et al., 2013). Genetic lineage tracing has revealed that the Tbx3\(^{+}\) SAN primordium forms along with the SV between E9-9.5 and E11.5-12.5 by the addition of SV myocardium is added to the venous pole by the differentiation of Tbx18\(^+/\)Nkx2-5\(^{-}\) progenitors (Christoffels et al., 2006; Mommersteeg et al., 2007; Wiese et al., 2009). The inflow tract cells of the E9.5 heart tube develop into atrial cells. Hcn4, initially expressed in the first-formed myocytes, is immediately activated in this Tbx18\(^+/\)/Nkx2-5\(^{-}\) domain and is downregulated in the Nkx2-5\(^{+}\) myocardium, thereby effectively shifting its expression domain to the newly added SV and the primary pacemaker domain (Garcia-Frigola et al., 2003; Mommersteeg et al., 2007; Liang et al., 2013; Später et al., 2013). Genetic lineage tracing has revealed that the Tbx3\(^{+}\) SAN primordium forms along with the SV between E9-9.5 and E11.5-12.5 by the addition of SV myocardium is added to the venous pole by the differentiation of Tbx18\(^+/\)Nkx2-5\(^{-}\) progenitors (Christoffels et al., 2006; Mommersteeg et al., 2007; Wiese et al., 2009). The inflow tract cells of the E9.5 heart tube develop into atrial cells. Hcn4, initially expressed in the first-formed myocytes, is immediately activated in this Tbx18\(^+/\)/Nkx2-5\(^{-}\) domain and is downregulated in the Nkx2-5\(^{+}\) myocardium, thereby effectively shifting its expression domain to the newly added SV and the primary pacemaker domain (Garcia-Frigola et al., 2003; Mommersteeg et al., 2007; Liang et al., 2013; Später et al., 2013). Genetic lineage tracing has revealed that the Tbx3\(^{+}\) SAN primordium forms along with the SV between E9-9.5 and E11.5-12.5 by the addition of
Tbx18+/Isil+/Nkx2-5- cells to the venous pole of the heart tube (Sun et al., 2007; Mommersteeg et al., 2007, 2010; Wiese et al., 2009). By E10, the SV and SAN have diverged from Nppa+ embryonic atrial cells (Hoogaars et al., 2007). The expression of Hcn4 becomes restricted to this Tbx3+ domain with further development (Fig. 3A). During the foetal period, the primary pacemaker site presumably shifts from the SV to the SAN (Vicente-Steijn et al., 2010), whereas the remainder of the Tbx3+ SV adopts an atrial phenotype and forms the right and left superior caval veins and the sinus venarum (Mommersteeg et al., 2007).

In chick embryos, SAN cells have been traced back to a small population of right lateral plate mesodermal progenitor cells posterior to the Nkx2-5+ heart field in HH stage 5 embryos. Cells from this region display pacemaker-like action potentials as early as HH stage 8, indicating early specification of pacemaker cell fate (Bressan et al., 2013).

Transcriptional programming of the SAN

The first molecular insights into SAN formation were gained only a decade ago, via a series of studies addressing SAN development in mice deficient for Tbx3, Tbx5, Tbx18, Shox2, Nkx2-5 and Pitx2 (Mori et al., 2006; Christoffels et al., 2006; Hoogaars et al., 2007; Mommersteeg et al., 2007; Blaschke et al., 2007; Wiese et al., 2009; Espinoza-Lewis et al., 2009). Together, these findings provided valuable information about where these transcription factors are expressed within the developing heart and how they affect SAN development and function (Fig. 3).

The transcription factor Tbx5 plays a key role in SAN formation. It is expressed in the SV and atra throughout development and interacts with Nkx2-5 to regulate the expression of short stature homeobox transcription factor 2 (Shox2), Tbx3 and Bmp4 – key regulators of SAN programme (Mori et al., 2006; Puskaric et al., 2010; Fig. 3). Shox2 is specifically expressed in the SV and is required for early SAN development and function (Blaschke et al., 2007; Espinoza-Lewis et al., 2009; Puskaric et al., 2010). In cultured embryonic stem cells (ESCs), Shox2 directs differentiation towards a pacemaker-like phenotype (Ionta et al., 2015). In vivo, Shox2 suppresses the working myocardial gene programme via repression of Nkx2-5 (Espinoza-Lewis et al., 2011). Deletion of Shox2 leads to a hypoplastic SV, upregulation of Nkx2-5 and, consequently, downregulation of Hcn4 and Tbx3 in the SAN primordium and in ESCs (Blaschke et al., 2007; Espinoza-Lewis et al., 2009; Hashem et al., 2013). Consistently, the loss of the pacemaker gene programme leads to conduction defects in Shox2-deficient mice (Blaschke et al., 2007; Espinoza-Lewis et al., 2009; Ye et al., 2015).

Tbx3 is expressed in the SAN but not in the atria or remainder of the SV, and it acts as a major regulator of the SAN gene programme by actively repressing factors within the working myocardial gene programme, including Cx40, Cx43, Scn5a and Nppa/b, to prevent atrialization (Fig. 3). Ectopic Tbx3 expression leads to the formation of bona fide functional pacemaker cells within these atria (Hoogaars et al., 2007; Bakker et al., 2012). Tbx3 thus imposes a pacemaker phenotype on cells within its expression domain. In the adjacent developing atrial myocardium, Nkx2-5 represses Tbx3 and Hcn4 expression. In line with this, Nkx2-5-deficient embryos, which die before E10, display ectopic Tbx3 and Hcn4 expression in the heart tube (Mommersteeg et al., 2007), whereas ectopic Nkx2-5 expression in cardiomyocytes, including those in the SAN, suppresses SAN formation (Espinoza-Lewis et al., 2011). This suggests that Nkx2-5 restricts the expression domains of Tbx3 and Hcn4 to the Nkx2-5- SV. The absence of Nkx2-5 in the SAN but not in other cardiomyocyte subtypes provides a useful tool for the identification of SAN cells in differentiated human ESCs. Human ESCs differentiated towards the cardiac lineage (using MYC and activation of fibroblast growth factor and bone morphogenetic protein signalling) yield both Nkx2-5+ chamber-like myocytes and Nkx2-5- pacemaker cells (Birket et al., 2015).

The transcription factor Pitx2, which determines the left-right asymmetry of internal organs, including the heart in vertebrates (Franco et al., 2014), is also involved in SAN development. In Pitx2-deficient embryos, SAN formation is observed at both the left and right sinoatrial junctions (Mommersteeg et al., 2007). The ectopic left SAN site expresses a SAN-like gene programme that includes Tbx3. Pitx2 directly represses Shox2 to mediate its transcriptional repression in the left side of the SV, and it positively regulates the expression of microRNAs that repress SAN genes, such as Shox2 and Tbx3, in this region (Wang et al., 2014). Pitx2 thus acts as a mediator of the right-sided development of the SAN by suppressing a SAN gene programme in the left SV (Fig. 3).

Tbx18 is expressed in all SV (including SAN) progenitor cells and in the subsequently formed sinus horns and head of the SAN (Christoffels et al., 2006; Wiese et al., 2009), and Tbx18 deficiency in mice leads to a malformed and strongly hypoplastic SV and SAN. However, Tbx18-deficient foetuses do not show obvious bradycardia, and the SAN gene programme is maintained.

Fig. 3. Development of the SAN. (A) Schematic of key factors expressed in the developing SV and SAN of mouse embryos. (B) Scheme depicting the roles of and the genetic interactions between factors involved in SAN formation. The dashed line indicates indirect activation of the SAN gene programme by Shox2. LA, left atrium; PV, pulmonary vein; RA, right atrium; SAN, sinoatrial node; SV, sinus venosus; WM, working myocardium.
in their hypoplastic SAN (Wiese et al., 2009), suggesting that Tbx18 does not directly regulate the SAN gene programme but is required for correct morphogenesis and deployment of the progenitor cells. Nevertheless, virally expressed Tbx18 suppresses the expression of Cx43, but not Cx40 and Cx45 (Gjc1), in ventricular myocytes (Kapoor et al., 2011). In the ventricles of pigs and guinea pigs, it leads to the ‘reprogramming’ of ventricular myocytes to pacemaker myocytes and ectopic pacemaker activity, accompanied by suppression of Cx43 and Nppa (also known as Anf), and upregulation of Hcn4 (Kapoor et al., 2013; Hu et al., 2014). We speculate that the differences between the loss and gain of Tbx18 function experiments can be explained by assuming that Tbx18, which is primarily a repressing T-box factor (Farin et al., 2007), mimics Tbx3 function when overexpressed (Fig. 3).

Recently, the LIM-homeobox transcription factor Isl1 has also gained attention as an important regulator of SAN development (Hoffmann et al., 2013; Liang et al., 2015; Vedantham et al., 2015). Isl1 is transiently expressed in, and is required for, cardiac mesodermal progenitors, and is downregulated as soon as they differentiate to cardiomyocytes. However, its expression is selectively maintained in myocytes of the SAN, both during embryogenesis and in the adult (Sun et al., 2007; Mommersteeg et al., 2010; Sizarov et al., 2011; Weinberger et al., 2012; Vedantham et al., 2015; Liang et al., 2015). In zebrafish, Isl1 marks pacemaker cells in the junction of the sinus venosus and atrium, and it is required for normal pacemaker function and development (Tessadori et al., 2012). In mouse, Isl1 is required for the proliferation and function of SAN cells, and the SAN-specific deletion of Isl1 results in embryonic lethality (Liang et al., 2015). Furthermore, Isl1-deficiency in mice leads to a downregulation of key regulators of SAN development, such as Tbx3, Shox2 and Bmp4, and ion channels for SAN function, including Hcn4, Hcn1 and Cacna1g (Liang et al., 2015; Vedantham et al., 2015), whereas Isl1 overexpression in ESC-derived cardiomyocytes leads to upregulation of the SAN gene programme and downregulation of the chamber myocardium gene programme (Dorn et al., 2015). Isl1 is a target of Shox2 in the SAN and can rescue the bradycardia phenotype caused by Shox2 deficiency (Hoffmann et al., 2013).

The origin and development of the AVC and AVN

The AVC can be first distinguished morphologically around E9, when the myocardium of the adjacent future atria and ventricles activates the working myocardium gene programme and starts to expand. Bmp2, Tbx2 and Tbx3 are among the first markers of the AVC (Hoogaars et al., 2004; Singh et al., 2012). Fate mapping by lipophilic dye labelling showed that the left posterior SHF of the 4-6 somite embryo (E8-8.5) contributes to superior AVC, whereas the right posterior SHF contributes to inferior AVC (Dominguez et al., 2012). Genetic lineage tracing has revealed that the AVC is derived from Tbx2+ cells of the IFT of the early tubular heart (Aanhaanen et al., 2009). Although the precise cellular origin of AVN myocytes has been a topic of debate, the current view is that the embryonic AVC contains the majority of precursors for the AVN and AV ring bundles (Aanhaanen et al., 2009; Vicente-Steijn et al., 2011).

The embryonic AVC retains a slow-conducting phenotype as a result of intrinsic myocardial gene programmes that actively repress chamber myocardial genes. In addition, the deposition of cardiac jelly at the onset of AV valve formation physically separates the AVC myocardium from the endocardium, preventing Cx40-induced endocardial-derived cues from reaching AVC myocytes (Bressan et al., 2014). From approximately E12 onwards, epicardial mesenchyme penetrates the myocardium between the AVC and ventricular myocardium and contacts the endocardium-derived cushion/valve mesenchyme to form the annulus fibrosus (Wessels et al., 1996; Zhou et al., 2010; Lockhart et al., 2014), thus insulating the atrial and ventricular working myocardium. Tbx3+ cells of the AVC form the definitive AVN and AV ring bundles at the atrial side of the annulus fibrosus. The atrium and ventricle are now electrically separated as the myocardial connection between both chambers is lost. The only conducting myocardial connection that remains between atrium and ventricle is the AVB, which forms from cells of the ridge of the ventricular septum and at the dorsal side remains in contact with the AVN primordium in the AVC.

Transcriptional regulation of AVC and AVN development

The AVC acquires its phenotype via a gene regulatory network that suppresses differentiation, stimulates nodal development, and strictly delimits the border between the AVC and chamber myocardium (Fig. 4). The assessment of the differential gene expression profiles of embryonic and foetal AVC and chamber myocardium indicated that, although the late foetal AVC/AVN largely maintains the embryonic AVC gene programme, the AVC undergoes substantial differentiation during development (Horshuis et al., 2009). Furthermore, the embryonic and foetal AVC express a number of neuronal genes not expressed in the chamber myocardium.

Key players in the networks underlying AVC development are Tbx2 and Tbx3, which have overlapping and partly redundant functions (Fig. 4). Tbx2 and Tbx3 act by repressing the expression of factors in the chamber myocardium gene programme, including Nppa, Cx40 and Scn5a, to retain the primitive phenotype of slow
conduction (Christoffels et al., 2004a; Harrelson et al., 2004; Aanhaanen et al., 2011; Singh et al., 2012). Furthermore, these T-box factors form a positive feedback loop with Bmp2 (Rutenberg et al., 2006; Singh et al., 2012). The inactivation of Tbx2, either globally or specifically in the developing myocardium, thus results in malformation of the annulus fibrosus and the ectopic expression of chamber genes in the AVC, leading to the formation of ectopic conductive AV pathways, reminiscent of the situation observed in Wolff–Parkinson–White syndrome of ventricular pre-excitation (Aanhaanen et al., 2011). Tbx2 and Tbx3 interact with the muscle-segment homeobox transcription factor Msx2, which is expressed in the AVC, to suppress the expression of Cx43 directly (Boogerd et al., 2008; Chen et al., 2008). They also compete with Tbx5 both for binding to T-box elements in target genes, such as Nppa and Cx40 (Hoogsaars et al., 2004; van den Boogaard et al., 2012), and for interaction with Nkx2-5 to repress Nppa in the AVC (Habets et al., 2002).

Bone morphogenetic protein 2 (Bmp2) is expressed specifically in the embryonic AVC and is required for AVC development, acting to control the AVC-restricted expression of Tbx2 and Tbx3 (Ma et al., 2005; Singh et al., 2012). Accordingly, AVC-restricted deficiency of the Bmp receptor Alk3 (Bmpr1a) results in defective AVN morphogenesis (Gaussin et al., 2005; Stroud et al., 2007). Bmp2/Smad signalling activates Tbx2 to drive its expression in the AVC (Ma et al., 2005; Singh et al., 2009); Tbx20, which is required for heart tube formation and chamber development, represses this activation to confine the expression of Tbx2 to the AVC and delimit the AVC boundary (Singh et al., 2009). In the zebrafish heart, the expression of tbx2b in the AVC is driven by the transcription factor encoded by foxn4 (also known as sli) (Chi et al., 2008), although a homologous murine Foxn4-mediated mechanism has not been established.

The boundary between the AVC and chambers is further delimited by Notch signalling. In the chick chamber myocardium, Notch signalling activates Hey1 and Hey2, which repress the expression of Bmp2. Conversely, Bmp2-activated Tbx2 represses the expression of Hey1 and Hey2 in the AVC, providing a feedback loop that sharpens the AVC boundary (Rutenberg et al., 2006). In mice, Hey1 and Hey2 prevent the expression of Tbx2 in the atrial and ventricular myocardium, respectively, although Hey1 and Hey2 expression is not affected by ectopic Tbx2 or Notch2 overexpression or by Notch2 inactivation (Kokubo et al., 2005; Rutenberg et al., 2006). The inhibition of Notch signalling in mice leads to a hypoplastic AVN and a disrupted AV nodal delay whereas, conversely, myocardial activation of Notch produces accessory pathways and ventricular pre-excitation (Rentschler et al., 2011). Canonical Wnt signalling is also required for correct AVC development and electrical programming. In zebrafish, Wnt signalling is required for the activation of bmp4 and tbx2b expression in the AVC (Verhoeven et al., 2011). Furthermore, in mice, the myocardial loss of Wnt signalling results in a hypoplastic right ventricle, which is associated with the loss of AVC myocardium, and the ectopic activation of canonical Wnt in the developing ventricle leads to a striking AVC phenotype in the ventricular wall (Gillers et al., 2015).

Finally, the GATA transcription factor family members Gata4 and Gata6, which play multiple roles in cardiac development, have also been implicated in AVC-specific gene expression and AV/C AVN development (Zhou et al., 2012; Stefanovic et al., 2014; Stefanovic and Christoffels, 2015). Gata4 heterozygous mutant mice have shortened PR intervals, suggesting that Gata4-mediated regulation of slow AV conduction target genes contributes to proper AV delay (Munshi et al., 2009). Together with Tbx5, Gata4 mediates the expression of Cx30.2 (Gjd2), which encodes a low conductance gap junction subunit, in the AVC/AVN (Munshi et al., 2009). Conversely, the basic helix-loop-helix (bHLH) transcription factor MyoR (musculin; Msc) represses the Gata4-dependent activation of Cx30.2, thereby fine-tuning the establishment of normal AV delay (Harris et al., 2015). Gata4 and Gata6 also regulate the expression of the transcriptional repressor Id2 and the cardiac sodium-calcium exchanger Ncx1 (Slc8a1), an important factor for the proper function of cardiac pacemaking (Lim et al., 2008; Liu et al., 2015). The myocardial-specific deletion of Gata6 leads to the downregulation of both of these genes, as well as downregulation of Hcn4, reduced cell-cycle exit of Tbx3+ cells leading to fewer Tbx3+ cells in the AVN, and PR interval prolongation (Liu et al., 2015).

**Development of the ventricular conduction system**

The ventricular conduction system (VCS), which makes up only 1% of the total muscle mass of the ventricles (Miquerol et al., 2010), comprises the AVB (or bundle of His), the left and right BBs running from the crest of the septum towards the apex, and the Purkinje fibre network. The VCS is of myocardial origin (Gourdie et al., 1995; Cheng et al., 1999). The distal BB and Purkinje fibre network form a thin layer of specialized myocytes just below the endocardium. The VCS shares a gene profile and some aspects of the nodal phenotype (e.g. poor contractile apparatus, fewer sarcomeres, more glycogen, latent automaticity) with the SAN and AVN. However, in contrast to the nodal myocardium, the VCS myocardium conducts the impulse rapidly and expresses high levels of Cx40 and Scn5a, which enable fast conduction of the impulse from the AVN to the ventricular working myocytes. Cx40 is the best characterized and most specific known marker for VCS cells; it is not expressed by the AVN or the ventricular working myocardium. Other specific and useful VCS markers that have been identified in mice are Irx3 (Christoffels et al., 2000; Zhang et al., 2011), Hcn4 (after birth; Liang et al., 2013), CCS-lacZ (Rentschler et al., 2001) and Cntn2-EGFP (Pallante et al., 2010). Furthermore, Tbx3 is selectively expressed in the AVB and BB, but not in the Purkinje fibre network (Hoogsaars et al., 2004).

The cellular origin of the VCS has been fairly well established, although some important details are still obscure. A domain in the AVC positive for G1N2 and Tbx3 expression extends dorsally and ventrally into the ventricular compartment and runs through the ventricular septum crest, forming the interventricular ring from which the AVB is formed (Wessels et al., 1992; Hoogaars et al., 2004). It is directly connected to the AVC-derived AVN, but the respective progenitors for these two tissues segregate before the onset of Tbx2 expression (Aanhaanen et al., 2009) in AVC precursors and before the activation of a Mef2c enhancer (as marked by a Mef2c-AHF-enhancer-Cre; Verzi et al., 2005) in septum precursors, at approximately E8-9 (Aanhaanen et al., 2010). The location of AVB (septum crest) progenitors in the embryo prior to ventricle formation has not been assessed. The BBs form from subendocardial myocytes of the trabecules of the septum. These components are present only in mammals and birds, and not in reptiles, amphibians and fish that lack a (complete) septum. The Purkinje fibre network is also found only in birds and mammals. It arises from the trabecular myocardium; during embryogenesis, the embryonic ventricles are largely composed of trabecular myocardium that acts as both the functional equivalent and cellular precursor of the Purkinje fibre network. It expresses Cx40, Irx3 and other VCS markers and rapidly conducts the impulse (Rentschler et al., 2001; Miquerol et al., 2010; Zhang et al., 2011).
2011). In fish, amphibians and reptiles, the Cx40+ trabecular myocardial wall is maintained into adulthood. In contrast to mammals and birds (Fig. 5A), these ‘lower’ endothermic vertebrates do not develop a massive Cx40-negative compact ventricular wall. As such, the trabecular myocardium is also likely to be the evolutionary precursor of the Purkinje fibre system of the endotherms (Jensen et al., 2012).

The spatiotemporal expression pattern of Cx40 suggests that the Cx40+ ‘Purkinje-like’ embryonic trabecular myocardium is the precursor of both the Cx40-negative compact ventricular working myocardium and the Cx40+ definitive Purkinje fibre network (Christoffels and Moorman, 2009; Fig. 5A). Elegant clonal and lineage analyses, based on the labelling and tracing of Cx40+ cells, have provided proof for this assumption (Miquerol et al., 2010, 2013). When Cx40+ cells are irreversibly pulse labelled at E10.5, when the still-thin trabecular left ventricular wall is entirely positive for Cx40 and the right ventricular wall is only partially so, labelled descendants are found both in the Purkinje fibres and in the compact wall. However, when cells are labelled at E16.5, when the Cx40-negative compact wall has already formed and the Cx40+ trabecular zone has become a relatively small component, descendants of Cx40+ cells are found only in the Purkinje network, indicating that the lineages of the working myocardium and Purkinje fibres have separated by E16.5.

**Molecular programming of the VCS**

Insights into the molecular mechanisms underlying trabecular development have shed light on the developmental mechanisms controlling the formation of the VCS, the product of the trabecular myocardium. Four key signalling factors or pathways have been implicated in trabecular development: Notch, Nrg1/ErbB (Egfr), ephrin B2/EphB4 and Bmp10 (reviewed by de la Pompa and Epstein, 2012; Fig. 5B). A model has thus been proposed in which Notch-mediated endocardial-myocardial interactions promote the transition of early embryonic ventricular myocardium into trabecular myocardium in an ephrin B2- and Nrg1-dependent manner; in this model, trabecular cardiomyocyte proliferation is sustained by Bmp10. Consistently, the administration of Nrg1 to cultured embryonic hearts isolated from the CCS-lacZ reporter line, in which the early trabecular myocardium in the embryonic heart and subsequently the VCS in the mature heart is marked by β-galactosidase expression, results in stimulation of lacZ expression. Moreover, the pattern of activation in the ventricles of cultured embryos changes (Rentschler et al., 2002). Together, these data suggest that Nrg1 stimulates the conduction phenotype of the trabecular myocardium. Similarly, Notch activation in embryonic ventricular myocardium results in stimulation of the conduction phenotype in adults, i.e. induction of Cntn2, Hcn1, Scn5a and CCS-lacZ expression, and in a cellular action potential change towards that of Purkinje fibre cells (Rentschler et al., 2012). In addition, transient Notch activation in neonatal ventricular myocytes using viral transduction causes induction of a Purkinje-like gene programme and electrophysiology.

The vascular cytokine endothelin has also been implicated in VCS development. During avian heart development, endothelin 1 regulates functional maturation of the VCS and activation of Cx40, and it has been suggested to be sufficient for the conversion of ventricular cardiomyocytes to Purkinje fibre cells (Gourdie et al., 1998; Kanzawa et al., 2002). However, mice harbouring deletions of both Ednra and Ednrb (which encode the two endothelin receptors expressed in mice) are viable, display no altered CCS gene expression and exhibit no obvious effects on CCS function (Hua et al., 2014). In addition, the administration of endothelin 1 to CCS-lacZ reporter mice did not change CCS-lacZ expression (Rentschler et al., 2002). The notion of endothelin 1-mediated conversion of ventricular myocytes into Purkinje fibre cells in chicken is also difficult to reconcile with the model described above, in which Cx40+ myocytes of the embryonic trabecular ventricle give rise to Cx40+ Purkinje fibre cells and Cx40− ventricular myocytes in the compact wall. Thus, although endothelin 1 signalling might stimulate Cx40 expression in chick embryos, it is not sufficient to induce conversion or lineage specification of the Purkinje fibre network (Christoffels and Moorman, 2009). Of note, in addition to the subendocardial Purkinje fibre network, bird hearts possess a Cx40+ Purkinje fibre network around the coronary arteries (periartrial), which might underlie the different interpretations regarding endothelin signalling and VCS differentiation.

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**Fig. 5. Development of the VCS.** (A) Expression of Cx40 in the ventricles from E9.5 to adult. Cx40 is expressed transmurally at E9.5. After E11.5, a Cx40-negative compact layer forms and expands at the epicardial side, whereas the trabecular component retains the same thickness throughout development. After birth, a further maturation step takes place, remodelling the trabecular zone into the definitive VCS. Scale bars: 100 µm. (B) Endocardium-myocardium interactions mediate the proliferation and differentiation of cells within the Cx40+ trabeculae (blue). Notch, Nrg1/ErbB and ephrin B2 signalling pathways mediate differentiation of the trabeculae (yellow arrows), whereas Bmp10 sustains trabecular cardiomyocyte proliferation (black arrows). (C) Scheme depicting the roles of and genetic interactions between factors involved in atrioventricular bundle (AVB) and bundle branch (BB) formation; cw, compact wall; lv, left ventricle; Pf, Purkinje fibres; tr, trabeculae.
Whereas the signalling factors described above are involved in controlling general VCS formation, a transcriptional network involving Nkx2-5, Tbx5, Tbx3, Irx3, Hopx and Id2 appears to control AVB and BB development and homeostasis (Fig. 5C). Based on data from mutant mice, a simple model can be formulated. Tbx3 is also expressed specifically from the earliest stages in the primordial AVB and BB, and it directly suppresses the working myocardial gene programme (including Cx40 and Cx43) and (indirectly) stimulates the pacemaker gene programme (e.g. Hcn4). Tbx5 is expressed more broadly in the ventricle, but in the AVB and BB it stimulates the gene programme for fast conduction, including Cx40 and Scn5a (Moskowitz et al., 2004; Arnolds et al., 2012). Here, it successfully competes with Tbx3 for occupation of binding sites of ‘conduction genes’ such as Cx40 and Scn5a. This leads to strong Scn5a expression in the AVB and BB from the outset (Remme et al., 2009), and to induction of Cx40 expression in the AVB during the foetal period, which seems to correlate with the AVB acquiring its function to primarily conduct the impulse. In embryos deficient for the bHLH transcription factor Hey2, the transmural expression of Tbx5, Cx40 and Scn5a, which are enriched in the trabecular component of the developing ventricle, is expanded into the compact myocardium (Xin et al., 2007; Koibuchi and Chin, 2007; Fischer et al., 2005; Bezzina et al., 2013). Hey2 is a transcriptional repressor, suggesting that it suppresses the expression of these genes in the compact wall, thereby contributing to the formation of the Purkinje fibre network. Nkx2-5 has a more complicated role, as it cooperates with both Tbx3 and Tbx5 and other factors. Nkx2-5 heterozygous mouse mutants display a prolonged QRS duration and low amplitude of AVB depolarization (Jay et al., 2004; Moskowitz et al., 2007). Furthermore, Nkx2-5 haploinsufficiency in mice results in severely hypoplastic Purkinje fibres and upregulation of Bmp10. Interestingly, although the trabecular myocardium in these mutants appears normal during development, normal Nkx2-5 levels were found to be required in a cell-autonomous manner for peri/postnatal maturation of the Purkinje fibres (Meysen et al., 2007). Purkinje fibre hypoplasia in Nkx2-5 mutants was rescued by Proxl haploinsufficiency (Risebro et al., 2012). In Tbx5/Nkx2-5 compound heterozygous mutants, the AVB and BB do not develop, and Id2 is not activated. Consistently, homozygous Id2 mutants fail to develop an AVB (Moskowitz et al., 2007). Given its function in other contexts, Id2 might also be involved in suppressing the working differentiation programme in the AVB and BB.

A number of additional factors have been implicated in VCS development. The homeobox transcription factor Irx3 is expressed in the trabecular ventricular myocardium and its expression becomes confined to the VCS (Christoffels et al., 2000; Zhang et al., 2011). Here, Irx3 directly suppresses Cx43 and (indirectly) activates Cx40 and Scn5a (Zhang et al., 2011; Koizumi et al., 2015). Irx3 mutant mice develop delayed ventricular activation and abnormal conduction (Zhang et al., 2011) and ventricular arrhythmias (Koizumi et al., 2015). In humans, sequence analysis of IRX3 exons in 130 probands of idiopathic ventricular fibrillation revealed two novel IRX3 mutations (Koizumi et al., 2015). The related factor Irx5 is expressed in the endocardium and subendocardial myocytes (including the layer that will form the Purkinje fibres) where it represses, amongst others, the transient outward potassium current (Ito) (Costantini et al., 2005). The homeobox protein Hopx also seems to play a role. In Hopx mutant mice, Cx40 expression is reduced in the AVB and BB. These mice show conduction defects corresponding to defective conduction below the AVN, including a wider QRS complex, longer QT interval and wider P wave (Ismat et al., 2005). Furthermore, Hf1b (Sp4) mutants display a sudden cardiac death phenotype caused by conduction defects and ventricular arrhythmogenesis, associated with a disturbed expression of Cx40 and Cx43 (Nguyen-Tran et al., 2000; Hewett et al., 2005).

**Regulatory elements in the CCS transcriptional network**

The identification of regulatory DNA elements that regulate gene activity in the CCS has provided further insight into the transcriptional mechanisms underlying CCS development and function. For example, characterization of the promoter region of Nppa in vivo led to identification of the Tbx2/Tbx3-mediated repression mechanism in the AVC (Habets et al., 2002). Cardiac Tbx3 expression is driven by two synergistically active distal enhancers that physically contact the Tbx3 promoter in the mouse embryo (van Weerd et al., 2014). One of these enhancers, located (~90 kb upstream of Tbx3, drives strong lacZ reporter expression in the ventral, right and dorsal portions of the AVC. The other enhancer drives pan-cardiac reporter expression without specific spatial information. When combined, both enhancers drive robust expression throughout the entire AVC and interventricular ring, indicating a mechanism in which the synergy of two regulatory modules regulates the expression of Tbx3 in atrioventricular conduction system (AVCS) precursors (van Weerd et al., 2014).

Tbx2 expression in the AVC is mediated through direct activation by Bmp2/Smad signalling. The 6 kb region directly upstream of Tbx2 is enriched for Smad-binding elements, and it contains regulatory sequences that are activated by Smads downstream of Bmp2-mediated signalling and are sufficient to recapitulate the expression of Tbx2 in the AVC and OFT (Kokubo et al., 2007; Shirai et al., 2009; Singh et al., 2009). In the chamber myocardium, the Bmp2-mediated activation of these sequences is perturbed by Tbx20, which physically interacts with and sequesters Smad1 and Smad5 (Singh et al., 2009). Furthermore, Hey1 and Hey2 suppress the Bmp2-mediated activation of these enhancers, further defining the strict boundary between atrium and AVC (Hey1) and ventricle and AVC (Hey2) (Kokubo et al., 2007). The activation of Cx30.2 by Gata4 and Tbx5 in the AVCS is also mediated by direct binding to a distal enhancer (Munshi et al., 2009). Conversely, Gata4 directly interacts with MyoR to bind and suppress this enhancer, thereby modulating a regulatory circuit that establishes AV delay (Harris et al., 2015).

The first regulatory element identified that consistently drives CCS-specific gene expression is the chicken Gata6 (cGata6)-enhancer, which in transgenic mice is active in the AVCS lineage (i.e. the AVC, AVB) from early embryonic stages onwards (Davis et al., 2001). Within the enhancer, a core unit of 47 bp is sufficient to restrict the expression of a reporter gene to the AVCS (Adamo et al., 2001). Within the enhancer, a core unit of 47 bp is sufficient to restrict the expression of a reporter gene to the AVCS (Adamo et al., 2001). Within the enhancer, a core unit of 47 bp is sufficient to restrict the expression of a reporter gene to the AVCS (Adamo et al., 2001). Within the enhancer, a core unit of 47 bp is sufficient to restrict the expression of a reporter gene to the AVCS (Adamo et al., 2001). Within the enhancer, a core unit of 47 bp is sufficient to restrict the expression of a reporter gene to the AVCS (Adamo et al., 2001).
Fig. 6. Regulatory elements and histone modifications involved in CCS development. (A) In the AVC (indicated by green arrow in the diagram below), GATA-binding enhancers recruit a Gata4-Smad4-Hat transcriptional activation complex, whereas a Gata4-Hey1,2-HDAC transcriptional repression complex is recruited in the chambers (indicated by red arrows in the diagram below). These establish AVC gene specificity in a coordinated manner. (B) Whole-mount fluorescence microscopy image of a double transgenic E10.5 embryo (left) and heart (right), in which the expression of green fluorescent protein (EGFP) is restricted to the AV canal and the expression of red fluorescent protein (Katushka) is restricted to the chamber myocardium. (C) ChIP-seq profiles of H3K27ac at Tbx2, Hey2 and Gata4 loci in EGFP+ (green), Katushka+ (red) or whole heart (black) cells. In AVC cells, H3K27ac marks, which are indicative of transcriptionally active regions, are enriched in the Tbx2 locus. By contrast, H3K27ac marks are enriched in the Hey2 locus in chamber cells. Gata4 is broadly expressed and its locus contains H3K27ac marks in both AVC and chamber cells. Images and diagrams reproduced from Stefanovic et al. (2014) with permission. AVC, atrioventricular canal; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.

chamber-specific transcriptional repressors Hey1 and Hey2 to deacetylate and repress the function of enhancers in the chambers (Stefanovic et al., 2014; Fig. 6B,C).

In the developing VCS, Id2 is cooperatively regulated by the binding of both Nkx2-5 and Tbx5 to a 1052-bp fragment of the Id2 promoter. Mutation of the Tbx5 binding site within this promoter region completely abolishes CCS-specific gene expression, whereas extracardiac expression is unaltered, illustrating the specificity of this transcriptional mechanism (Moskowitz et al., 2007). Enhancers have also been identified in the Scn5a/Scn10a locus; these drive VCS-specific gene expression and are controlled by Tbx5, Tbx3, Nkx2-5 and Gata4 (Arnolds et al., 2012; van den Boogaard et al., 2012).

Histone modifications mediating CCS-specific gene expression

As touched on above, HATs and HDACs, which play key roles in the regulation of gene expression by controlling DNA accessibility (Backs and Olson, 2006; Bruneau, 2010; Han et al., 2011; Chang and Bruneau, 2012), have been implicated in CCS development and function. For example, Hdac3, a member of the Class I HDAC family, has been implicated in cardiac development and homeostasis (Montgomery et al., 2008; Singh et al., 2011), acting by repressing the expression of Tbx5 in early development (Lewandowski et al., 2014). During development, it is highly expressed in the SAN (Wu et al., 2014), AVN and Purkinje fibres (Risebro et al., 2012). The prospero-related homeobox protein 1 (Prox1) recruits Hdac3 in the AVN and Purkinje fibres to repress Nkx2.5 directly through a proximal upstream enhancer, thereby controlling electrophysiological homeostasis in the adult heart (Risebro et al., 2012). In the SAN, too, Hdac3 is involved in Nkx2-5 repression. By direct interaction with Tbx3 and Baf250a (Ard1a), a key component of the SWI/SNF family of chromatin remodelling complex, a dynamic equilibrium of acetylation and deacetylation on the Nkx2-5 promoter leads to transcriptional repression in the SAN (Wu et al., 2014). Conversely, regulatory sequences upstream of Baf250a are co-occupied by Nkx2-5, Shox2 and Tbx5, suggesting a mechanism in which these factors mediate the expression of Baf250a in the SAN (Ye et al., 2015).

Hdac1 and Hdac2, other members of the Class I HDACs, have redundant roles in cardiac morphogenesis and growth. As mentioned before, they interact with Gata4 to regulate the acetylation state of AVC-specific enhancers, thereby playing a crucial role in the AVC-specific expression and differentiation of AVC versus working myocardium (Stefanovic et al., 2014). Their crucial role in the development of the CCS is furthermore underlined by the observation that myocardial-specific deletion of both Hdac1 and Hdac2, but not either of them alone, results in the upregulation of calcium channel genes, including Cacna1h and Cacna2d2 (Montgomery et al., 2007). Cacna1h (also known as Cav3.2) is a T-type calcium channel expressed in the embryonic heart but downregulated after birth (Yasu et al., 2005). Cacna2d2, the L-type calcium channel subunit α2δ2, is specifically expressed in the nodal tissues of the embryonic and adult heart (Marionneau et al., 2005; Singh et al., 2012). HDAC activity thus spatially restricts the activity of these genes by imposing a suppressive histone modification on these genes in non-CCS cells, presumably involving the AVC enhancer-switch mechanism described above (Stefanovic et al., 2014). Class Ila HDACs also mediate the function of an intronic contiguous region of genomic DNA within Hcn4 that acts as enhancer and is active in the embryonic AVC and VCS portion of the Hcn4-expression domain. In the adult heart, this...
enhancer is active in the compact AVN and AVB. The inhibition of HDAC activity by trichostatin A (TSA) leads to expansion of the enhancer activity domain throughout the entire heart (Vedantham et al., 2013), consistent with the model of HDAC-mediated suppression of CCS-specific enhancers in the chamber myocardium.

**Genomic variation affects CCS function**

In humans, haploinsufficiency for the cardiac core transcription factors, including TBX5 and NKX2-5, causes profound CCS dysfunction and arrhythmias (Basson et al., 1997; Li et al., 1997; Schott et al., 1998). The analysis of Tbx3 hypomorphic and conditional mouse mutants with varying levels of Tbx3 expression within the heart revealed that CCS function and homeostasis is extremely sensitive to Tbx3 dosage (Frank et al., 2011). Minor perturbations in CCS regulatory networks can thus have significant effects on CCS development or function and lead to a predisposition for CCS dysfunction. Knowledge of the apparent tight transcriptional control of CCS genes is therefore crucial to fully understand the phenotypes caused by their misregulation.

Several genome-wide association studies (GWAS) have been conducted in the last decade, uncovering common variants within the human population that are associated with traits affecting CCS function, including perturbed PR interval, QRS duration and QT interval. The loci identified in these studies harbour genes encoding well-known cardiac transcription factors such as TBX3, TBX5, TBX20, NKX2-5, MEIS1 and HEY2, and genes encoding ion channels such as SCN5A, SCN10A, KCNQ1, KCNH2 and HCN4 (Holm et al., 2010; Pfeufer et al., 2010; Sotoodehnia et al., 2010; Bezzina et al., 2013; den Hoed et al., 2013; Verweij et al., 2014; Table 1). Interestingly, as discussed above, many of these genes are implicated in the CCS gene regulatory network. These findings suggest that the transcriptional network is highly sensitive to genetic variations that influence the balance of these factors. In addition, the majority of associated variants localize to non-coding genomic regions, suggesting that they affect regulatory sequences involved in controlling the expression level and pattern of target genes within the variant loci (Maurano et al., 2012; Sakabe et al., 2012). Although the effect of the associated variants is generally relatively small, they can have significant effects on CCS function and lead to predisposition for several disorders. For example, non-coding variants in the SCN5A-SCN10A locus and close to HEY2 have been associated with Brugada syndrome, a rhythm disorder with a high risk of sudden cardiac death (Bezzina et al., 2013). The current challenge now is to identify the functional elements in the implicated risk loci to uncover how they affect normal function of their target genes.

Table 1. Loci harbouring GWAS variants associated with ECG parameters

| Associated trait | Putative target genes associated with ECG parameters | Locus | Reference |
|------------------|--------------------------------------------------|-------|-----------|
| Heart rate       | NKX2-5 Transcription factor                      | 5q34  | den Hoed et al., 2013 |
|                  | CX40 Gap junction subunit                        | 6q21-23.2 | den Hoed et al., 2013; Eijgelshiem et al., 2010 |
|                  | HCN4 Potassium channel                           | 15q24.1 | den Hoed et al., 2013 |
|                  | MYH6, MYH7 Myosin heavy chain                    | 14q11.2 | Mizusawa and Wilde, 2012; den Hoed et al., 2013; Eijgelshiem et al., 2010; Holm et al., 2010 |
| PR interval      | NKX2-5 Transcription factor                      | 5q35.1 | Pfeufer et al., 2010; Holm et al., 2010; Verweij et al., 2014 |
|                  | TBX3, TBX5 Transcription factor                  | 12q24.21 | Pfeufer et al., 2010; Holm et al., 2010; Verweij et al., 2014 |
|                  | MEIS1 Transcription factor                       | 2p14  | Pfeufer et al., 2010 |
|                  | SOX5 Transcription factor                        | 12p12.1 | Pfeufer et al., 2010 |
|                  | WNT11 Signalling protein                         | 11q13.5 | Pfeufer et al., 2010 |
|                  | SCN5A, SCN10A Sodium channel                     | 3p22.2 | Holm et al., 2010; Pfeufer et al., 2010; Chambers et al., 2010 |
| QRS duration     | CAV1, CAV2 Calcium channel                       | 7q31.1 | Holm et al., 2010; Pfeufer et al., 2010 |
|                  | TBX3, TBX5 Transcription factor                  | 12q24.21 | Holm et al., 2010; Pfeufer et al., 2010 |
|                  | TBX20 Transcription factor                       | 7p14.3 | Sotoodehnia et al., 2010 |
|                  | HAND1 Transcription factor                       | 5q33  | Sotoodehnia et al., 2010 |
|                  | SCN5A, SCN10A Sodium channel                     | 3p22.2 | Holm et al., 2010; Chambers et al., 2010; Sotoodehnia et al., 2010 |
| QT interval      | CACNA1D Calcium channel                          | 3p14.3 | Sotoodehnia et al., 2010 |
|                  | TBX5 Transcription factor                        | 12.24q1 | Holm et al., 2010 |
|                  | SCN5A Sodium channel                             | 3p22.2 | Holm et al., 2010; Chambers et al., 2010; Pfeufer et al., 2009; Newton-Cheh et al., 2009 |
|                  | KCNQ1 Potassium channel                          | 11p15.5 | Holm et al., 2010; Newton-Cheh et al., 2009; Pfeufer et al., 2009 |
|                  | KCNJ2 Potassium channel                          | 17q24.3 | Holm et al., 2010; Chambers et al., 2010; Pfeufer et al., 2009 |
|                  | KCNH2 Potassium channel                          | 7q36.1 | Holm et al., 2010; Chambers et al., 2010; Newton-Cheh et al., 2009; Pfeufer et al., 2009 |
| Brugada syndrome | KCNE1 Potassium channel                          | 21q22.12 | Holm et al., 2010; Newton-Cheh et al., 2009 |
|                  | HEY2 Transcription factor                        | 6q22  | Bezzina et al., 2013 |
|                  | SCN5A, SCN10A Sodium channel                     | 3p22  | Bezzina et al., 2013 |
preferentially contact each other to regulate gene transcription (Lieberman-Aiden et al., 2009; Dixon et al., 2012). Hence, chromatin 3D structure and TAD organization provide important information regarding gene regulation. Taking these factors into consideration, we are beginning to gain insight into how certain variants might affect CCS function.

Variants in the **SCN5A-SCN10A** locus, for example, are associated with modulation of PR interval (Chambers et al., 2010; Holm et al., 2010; Pfeuffer et al., 2010; Smith et al., 2011), QRS duration (Holm et al., 2010; Sotoodehnia et al., 2010) and QT interval (Newton-Cheh et al., 2009; Pfeuffer et al., 2010; Sotoodehnia et al., 2010), which predispose to conduction and repolarization disorders. However, although a role for **SCN5A** in conduction and arrhythmias has been firmly established, a role for **SCN10A** in cardiac conduction came as a surprise and has been the subject of some controversy (Verkerk et al., 2012; Yang et al., 2012). Both **Scn5a** and **Scn10a** were found to be targets of **Tbx3** and **Tbx5** in vivo (van den Boogaard et al., 2012; Fig. 7A). One of the major risk variants within the **SCN5A-SCN10A** locus, the common SNP rs6801957, has been shown to affect a conserved T-box binding site in an intronic enhancer in **SCN10A** that is occupied by **TBX3** and **TBX5** and that drives activity in the VCS (van den Boogaard et al., 2014). Recent circular chromosome conformation capture sequencing (4C-seq) analyses revealed that the expression of **Scn5a**. **Scn10a** is activated by **Tbx5** in the AVB, but repressed by **Tbx3** in the SAN, AV/C and AVN myocardium, demonstrating the dominant-repressive function of **Tbx3** in these compartments. As the expression domain of **Scn5a** is wider than that of **Scn10a**, other factors are at play to activate **Scn5a** in the right ventricle. (B) The enhancer in **Scn10a** physically contacts both **Scn5a** and **Scn10a** to mediate their expression. (C) The variant in **Scn10a** alters **Scn5a** expression. The major allele harbours a T-box binding element that is occupied by **Tbx3** and **Tbx5** to drive **Scn5a** expression in the heart (indicated by black arrowheads). The variant alters a base in the minor allele, disrupting the T-box binding element and causing a strongly decreased affinity for **Tbx3** and **Tbx5**. This results in the loss of enhancer activity, hence the loss of **Scn5a** expression (indicated by white arrowheads).

Such GWAS-based analyses have also provided information about how **Tbx** genes are regulated in the CCS. The genes encoding **Tbx3** and **Tbx5** are organized in an evolutionarily conserved cluster (Agulnik et al., 1996) and their overlapping expression patterns and function during development suggest co-regulatory mechanisms for their transcriptional control (Hoogaars et al., 2004). Variants in the gene desert flanking the cluster have been associated with prolonged PR interval and QRS duration (Holm et al., 2010; Pfeuffer et al., 2010; Sotoodehnia et al., 2010; Verweij et al., 2014), but the functional annotation of these variants remains to be elucidated. However, 4C-seq studies in embryonic mouse hearts revealed that both loci are organized in separate TADs and form individual chromatin loops, thereby physically separating the **Tbx3** loop from that of **Tbx5** (Fig. 8A) and rendering enhancer sharing between both genes unlikely (van Weerd et al., 2014). This is in contrast to the transcriptional regulation of the well-studied Irx and Hox developmental gene clusters, in which enhancer sharing occurs...
extensively, possibly underlying the conservation of their genomic organization during evolution (Duboule, 1998; Tena et al., 2011). Hi-C in human cells, probing contact profiles at a genome-wide level, confirmed that the separated domain organization of the TBX3-TBX5 locus is conserved between mouse and human (Jin et al., 2013). Such knowledge facilitates the assignment of function to variation, as it can be concluded that regulatory sequences affected by trait-associated variants located within the TBX3 locus are exclusively regulating TBX3 but not TBX5, and vice versa.

Conclusions
Our understanding of the developmental processes that drive CCS formation has greatly improved over the last decade. Key factors have been identified, unravelling the transcriptional networks underlying the formation of distinct CCS components. The numerous GWAS-based approaches that have recently been conducted on rhythm, conduction and repolarization parameters underlines the sensitive nature of the mechanisms governing CCS development, as they link common variants affecting factors crucial for CCS development and function to conduction traits. Although individual variants generally exert a relatively mild effect, they can affect sensitive regulatory pathways and lead to dysregulation of key factors in CCS development and function, and hence to a predisposition to conduction system disorders and arrhythmias. The main challenge that we are facing now is to link the associated variants with functional modules and investigate how their misregulation affects cardiac conduction. The emergence of novel techniques for assessing genome-wide transcription factor occupancy (ChIP-seq), chromatin topology (3C/4/5Hi-C-seq), chromatin accessibility (DNase-seq, ATAC-seq) and transcriptomes (RNA-seq) in specific cell types will no doubt provide indispensable insight.

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