A novel conditional mouse model for Nkx2-5 reveals transcriptional regulation of cardiac ion channels

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ABSTRACT

*Nkx2-5* is one of the master regulators of cardiac development, homeostasis and disease. This transcription factor has been previously associated with a suite of cardiac congenital malformations and impairment of electrical activity. When disease causative mutations in transcription factors are considered, *NKX2-5* gene dysfunction is the most common abnormality found in patients. Here we describe a novel mouse model and subsequent implications of Nkx2-5 loss for aspects of myocardial electrical activity. In this work we have engineered a new Nkx2-5 conditional knockout mouse in which flox sites flank the entire Nkx2-5 locus, and validated this line for the study of heart development, differentiation and disease using a full deletion strategy. While our homozygous knockout mice show typical embryonic malformations previously described for the lack of the Nkx2-5 gene, hearts of heterozygous adult mice show moderate morphological and functional abnormalities that are sufficient to sustain blood supply demands under homeostatic conditions. This study further reveals intriguing aspects of Nkx2-5 function in the control of cardiac electrical activity. Using a combination of mouse genetics, biochemistry, molecular and cell biology, we demonstrate that Nkx2-5 regulates the gene encoding *Kcnh2* channel and others, shedding light on potential mechanisms generating electrical abnormalities observed in patients bearing *NKX2-5* dysfunction and opening opportunities to the study of novel therapeutic targets for anti-arrhythmogenic therapies.

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INTRODUCTION

The homeobox transcription factor Nkx2-5 is the master regulator of cardiac specification and is essential for the maintenance of heart homeostasis in adults (Elliott et al., 2010). Originally identified in Drosophila and named tinman, as its mutation eliminated the dorsal vessel heart anlage, Nkx2-5 controls diverse aspects of a highly evolutionarily conserved transcriptional network (Clark et al., 2006). Mutations in the mammalian NkX2-5 gene are associated with severe structural and functional heart impairment and constitute one of the most common genetic causes of congenital heart disease (CHD) in humans, with an incidence of ~4% of all cases (Benson and Martin, 2010). Patients presenting heterozygous NkX2-5 mutations display diverse cardiac abnormalities that include septal defects, conotruncal malformations, hypoplastic left heart, dilated cardiomyopathy, atrioventricular conduction block (Costa et al., 2013; Elliott et al., 2003; Gutierrez-Roelens et al., 2006; Gutierrez-Roelens et al., 2002; Hirayama-Yamada et al., 2005; Kasahara and Benson, 2004; Schott et al., 1998), ventricular arrhythmia and sudden death (Perera et al., 2014). Mouse Nkx2-5 knockout models show impaired growth and chamber specification during embryonic development, associated with severe deregulation of the cardiac regulatory transcriptional network, leading to early embryonic lethality (Lyons et al., 1995; Tanaka et al., 1999). Heterozygous and conditional deletions of Nkx2-5 at postnatal stages have shown that its activity is also essential for ventricular myocyte maturation, maintenance of the cardiac conduction system and overall heart homeostasis (Biben et al., 2000; Briggs et al., 2008; Nakashima et al., 2014; Pashmforoush et al., 2004; Takeda et al., 2009; Terada et al., 2011). However, conditional models generated so far rely on partial deletion of the Nkx2-5 locus (Pashmforoush et al., 2004), therefore interpretation of phenotype in these mice is confounded by the generation of a smaller Nkx2-5 coding mRNA containing the important TN domain, known to interact with the repressor complexes (Elliott et al., 2010).

In humans, electrophysiological defects associated with NkX2-5 mutations can be directly linked to the cardiac conduction system, in particular atrioventricular node and proximal Purkinje fibers, or to the action potential of the working myocardium. Several independent groups have investigated conduction defects in Nkx2-5 mouse models where: 1. perinatal deletion of Nkx2-5 causes arrhythmia marked by a dysmorphic and smaller atrioventricular node and decreased expression of the voltage activated sodium channel Nav1.5 (Briggs et al., 2008); 2. atrial specific deletion leads to hyperplastic atria and conduction system (Nakashima et al., 2014) and 3. heterozygous deletion of Nkx2-5 results in decreased Purkinje fiber density (Jay et al., 2004; Meysen et al., 2007). Interestingly, one of the most common outcomes of impaired NkX2-5/Nkx2-5 gene activity in humans and mouse models used so far is the presence of increased arrhythmias and atrioventricular block. These data show that
the role played by this transcription factor in cardiac function is further complicated by temporal regulation and differential roles in distinct heart sub-compartments, justifying the need for the generation of a proper conditional deletion model for the study of the various roles of Nkx2-5 in mice. Given the extensive range of supporting evidence implicating Nkx2-5 in the control of cardiac electrophysiology in mice and humans, we used our novel Nkx2-5 mutant mouse model in which all exons (1 and 2) of the endogenous locus are fully removed to further investigate its role in the propagation of the cardiac action potential. We demonstrate that Nkx2-5 regulates a wide range of ion channels essential for heart physiology, pinpointing Kcnh2 and Cacna1C as novel targets associated with Nkx2-5 dysfunction. Following up on Kcnh2 regulation, we show that Nkx2-5 protein directly regulates Kcnh2 expression, a gene coding for the rapidly activating delayed rectifier potassium current (I_{Kr}), associated with Long Q-T syndrome type 2 (LQT2). Furthermore, we confirmed that Herg channel activity is also affected by Nkx2-5 mutant proteins with reduced transcriptional activity. Our results demonstrate a direct regulatory link between Nkx2-5 and Kcnh2, thereby uncovering novel molecular mechanisms for the generation of complex arrhythmic events identified in humans and mice with Nkx2-5 mutations.
RESULTS

Mice bearing a novel Nkx2-5 null allele show predicted impairment of heart development.

Given the multifaceted role of Nkx2-5 in controlling heart morphogenesis and homeostasis, it is not surprising to find complex phenotypes in patients and mouse models with perturbed Nkx2-5 protein activity. To investigate the consequences of the full deletion of Nkx2-5 locus, we generated a new Nkx2-5 conditional line (Nkx2-5 cKo). LoxP sites were inserted before the first exon and in the 3’ untranslated region of the Nkx2-5 gene (Fig. 1A-E and Supplementary Fig. 1). A PGK-Neo cassette flanked by FRT sites in the opposite direction was inserted at the 3’ end of the gene to allow selection for genome integration. Correct recombination was confirmed by Southern analysis (Fig. 1F). Our homozygous Nkx2-5 cKo mice show no significant difference in survival or fertility when compared to wild type or heterozygous littermates. It is still possible that our model displays a certain level of hypormorphism, as previously seen for several models where insertion of cassettes disrupted unknown regulatory regions of the gene, although we have not tested this possibility.

Full deletion of the Nkx2-5 gene (Nkx2-5Δ) was obtained by crossing the conditional line with CMV-cre (Fig. 1E), which displays germline expression of the cre-recombinase enzyme. Deletion was confirmed by PCR (Fig. 1G). Heterozygous Nkx2-5^5/Δ+ mice were viable and fertile but homozygous embryos (Nkx2-5^5/Δ) died around 10.5 days post-coitum (dpc), presenting hypoplastic hearts and looping abnormalities, similar to other previously described Nkx2-5 null models (Fig. 2A) (Lyons et al., 1995; Tanaka et al., 1999). qPCR showed a reduction of 50% of Nkx2-5 mRNA levels in 9.5 dpc heterozygous (Nkx2-5^5/Δ+) hearts compared to wild-type (WT) littermates, while Nkx2-5^5/Δ embryos had no detectable Nkx2-5 transcript or protein (Fig. 2B-C), confirming full genomic deletion. Expression of markers for the maturing working myocardium Nppa (atrial natriuretic factor) and Gja5 (connexin 40) were down-regulated in Nkx2-5^5/Δ embryos, while the pan-myocyte marker Mlc2v was still present, demonstrating that early cardiac specification is partially maintained (Fig. 2D). Interestingly, Gja5 was also found reduced in the vasculature, a site where Nkx2-5 is not expressed. This is most likely due to a secondary effect in vasculature patterning caused by impaired heart function.

Nkx2-5 mutant hearts show transcriptional dysregulation of ionic channel genes

To assess regulation of action potential genes by Nkx2-5, we performed qPCR for major cardiac ion channels in 9.5dpc isolated hearts, a stage in which heart beating was still observed in both control (WT, Nkx2-5^5/++) and mutant (Nkx2-5^5/Δ) embryos. Expression of several channels was significantly
affected in mutant embryos, most of them in a dose dependent manner (Fig. 3A-C). These included
the sodium channel Scn5a (coding for Nav1.5, I\textsubscript{Na} current – Fig. 3A) and the voltage-activated
calcium channel Cacna1c (coding for Cav1.2, I\textsubscript{CaL} current – Fig. 3B). No significant changes in
transcript levels were observed for the calcium-ATPase pump Serca2 transcripts (Fig. 3D).
Interestingly, a significant decrease in both heterozygous and homozygous embryos was also
observed in the expression level of the voltage-activated K channel Kcnh2 (coding for ERG
channels), so far not associated with Nkx2-5 function (Fig. 3C). This channel is essential for the late
repolarization phase in the action potential of atrial and ventricular cardiomyocytes, as well as for the
regulation of pacemaker activity in sinoatrial node cells (Clark et al., 2004). Mutations in this gene
have been associated with the long-QT syndrome (Curran et al., 1995).

**Nkx2-5 binds to the Kcnh2 promoter and directly regulates isoform merg1b.**

Due to the novel ionic channel regulation uncovered herein, we deepened our analysis to dissect
whether Kcnh2 is directly regulated by Nkx2-5 protein. In mice, the endogenous Kcnh2 locus
encodes 2 different isoforms, merg1a and merg1b. Both isoforms were down-regulated in Nkx2-5
mutant embryos, although the decrease in expression was more evident for the merg1b isoform (Fig.
4A), which is normally less abundant in the heart. Paradoxically, immunofluorescence analysis of
E9.5 embryo hearts showed no significant decrease in protein level, probably because the antibody
used in this study is unable to distinguish between the different isoforms (Supplementary Fig. 2).
Using bioinformatics mining of mouse ENCODE (Stamatoyannopoulos et al., 2012) and other
datasets (Sakabe et al., 2012; van den Boogaard et al., 2012) within the UCSC genome browser
(Karolchik et al., 2014) we identified putative Nkx2-5 binding sites in highly conserved regions of
the Kcnh2 channel genomic region, especially upstream of merg1b. In particular, a putative enhancer
containing a conserved binding site for Nkx2-5 (NKE) was localized to a 500 bp fragment between
positions 2398-2823 bp upstream of the merg1b transcription start site (Fig. 4B, grey box, distal site).
Another set of canonical Nkx2-5 binding sites was identified 1000 bp upstream of the transcriptional
start site for the same isoform (Fig. 4B, grey box, proximal site). The identified proximal putative
region overlaps with peaks of H3K4me1 and H3K27ac, markers of active chromatin enhancers,
while peaks of H3K4me3, H3K27ac and DNAse hypersensitivity sites (DHSs) were found at the
transcriptional initiation site of merg1b (Fig. 4B), suggesting the presence of a promoter element.
These Nkx2-5 proximal and distal binding regions have not been identified by a previous screening
for transcriptional enhancers in the heart (He et al., 2011). The distal putative regulatory region did
not display any significant signature of active chromatin. Interestingly, active chromatin markings in
the proximal region were not seen in other tissues, indicating the presence of a cardiac specific regulatory region (Supplementary Fig. 3).

To investigate whether Nkx2-5 directly regulates Kcnh2, we assessed the ability of Nkx2-5 protein to bind to both distal and proximal enhancer elements at the Kcnh2 promoter, using chromatin immunoprecipitation (ChIP) analysis (Fig. 4C). Both DNA elements showed specific binding to Nkx2-5 protein [Fig. 4C, sites proximal (P) and distal (D1)], albeit with lower strength than the bonafide Nkx2-5 sites on Nppa promoter. Another putative site present at the distal region (D2) showed no binding (Fig. 4C). We further demonstrated that Nkx2-5 can synergize with known cardiac transcription partners Gata4 and Tbx5, leading to robust activation of a 4.5 Kb promoter region of Kcnh2, isoform merg1b in COS-7 cells (Fig. 4D). Interestingly, deletion of the distal enhancer region (2398-2823 bp upstream of promoter) still allowed for strong luciferase activity, highlighting the importance of the proximal site P for transcriptional activation. This core proximal region also contained a putative site for Nkx2-5 co-partner Gata4 (Fig. 4E). To confirm the importance of the proximal NKE site for the regulation of merg1b, we generated 2 point-mutations at the core region, creating a Nkx2-5 mutant site previously shown to incapacitate protein binding (Linhares et al., 2004).

To test the regulation of Kcnh2 by Nkx2-5 in a more physiological scenario, we used HL-1 atrial cardiomyocytes. The full-length 4.5Kb merg1b regulatory region showed minimal activation in this system (Fig. 4E, right panel). Deletion of the distal element D or mutation of the proximal element P had no effect on transcriptional activity (Fig. 4E, red bars). On the other hand, deletion of the 3.5Kb 5’ region dramatically increased activation of the reporter gene (8-fold over promoterless construct; Fig. 4E, blue bar), suggesting the presence of a repressor element in the full (4.5Kb) construct external to D element. The mutation in the proximal NKE site led to a 50% decrease in reporter activity of this construct, consistent with an essential role for Nkx2-5 in merg1b transcriptional regulation (Fig. 4E; blue bar/white squares). The residual activation suggests the presence of other transcription regulatory units for Nkx2-5 and co-factors are still active in this region.

**Nkx2-5 mutant protein impairs Kcnh2 activity**

We have previously identified a NKX2-5 mutation associated with the development of dilated cardiomyopathy and conduction defects in patients (Nkx2-5 I183M - (Costa et al., 2013). In this study, there was complete correlation between the presence of the mutation in afflicted family members and the presentation of symptoms of conduction defects that included block of the right...
branch of the bundle of His (Costa et al., 2013). To study the consequence of Nkx2-5 dysregulation on Kcnh2 channel, we performed patch clamp in isolated HL-1 cells co-transfected with Nkx2-5 I183M and a red fluorescent reporter (Fig. 5A) to measure Kcnh2 channel current (ERG). HL-1 cells are known to be a reliable model to study heart function and electrophysiology, expressing most of the cardiac transcription factors (including Nkx2-5), ion channels and structural genes. Transfection with empty vector was used as internal control (White et al., 2004). Consistent with the predicted role of Nkx2-5 in regulating merg1b expression levels, transient expression of Nkx2-5 I183M mutant protein showed a marked reduction of the ERG current, when compared to cells expressing the empty vector (Fig. 5B-C). Specificity of measurement for ERG activity was confirmed by sensitivity to dofetilide (Fig. 5B, lower panels). Collectively, these data demonstrate that Nkx2-5 is essential for the regulation of ERG currents in cardiac cells. Disturbances of these currents, conferred by presence of Nkx2-5 mutant proteins, can cause significant electrophysiological dysfunction, suggesting a role for ERG currents in the complex arrhythmic changes observed in human patients presenting heterozygous mutations for NKK2-5. Nkx2-5 also directly regulates Scna5a mRNA levels, responsible for the rapid depolarizing Na⁺ current I_{Na⁺}(Fig. 3) and in agreement with this, I183M expression significantly reduced the peak amplitude and maximum rate of rise (MRR) of the action potential in HL-1 cells (Fig 5D-E).

Electrical abnormalities of Nkx2-5^{∆/+} hearts are compensated for in adulthood

As Nkx2-5^{∆/+} animals survive to adulthood, we tested whether the lack of one allele of this gene can cause notable abnormalities of heart function. Histological analysis showed mild dilation of ventricular chambers and right atrium under homeostatic conditions (Fig. 6A), while magnetic resonance imaging (MRI) measurements indicated that young Nkx2-5^{∆/+} adult mice do not display gross cardiac dysfunction, despite showing smaller left ventricular (LV) mass, diminished cardiac output and a tendency to have reduced LV ejection fraction (Fig. 6B, Suppl. Table 1). The reduction in these parameters most likely reflects the slightly lower body weight (BW) observed for heterozygous animals. When heart weight (HW) was normalised by BW, no significant differences were observed (Suppl. Table 1). ECG activity remained unchanged in non-anesthetized Nkx2-5^{∆/+} mice, indicating lack of gross arrhythmogenic abnormalities (Fig. 6C-D). Due to the high correlation of Nkx2-5 activity with septal dysmorphogenesis (Biben et al., 2000), we investigated the presence of atrial septal abnormalities in heterozygous Nkx2-5^{∆/+} mice. Patent Foramen Ovales (PFOs) were detected in approximately 23% (3/13 hearts) of WT mice, as expected for the C57BL/6 genetic background (Biben et al., 2000). This frequency was increased to 90% in Nkx2-5^{∆/+} mice (10/11 hearts). Furthermore, PFOs found in most Nkx2-5^{∆/+} mice (9/11) were larger and displayed wider flap
valves than WT cohorts, indicative of a more severe phenotype. No atrial septal defects (ASDs) were detected in either WT or $Nkx2-5^{+/+}$ mice. Collectively these data indicate that the absence of one $Nkx2-5$ allele is not overly detrimental to adult cardiac function in itself, although it causes moderate morphological and functional abnormalities, which could be aggravated by external factors like obesity and diabetes, as is the case in patients with sedentary lifestyle.

Considering the subtle changes in the overall function of $Nkx2-5^{+/+}$ adult hearts, we examined whether important channels detected in early embryonic development were similarly expressed in normal adult hearts (Fig. 7). Strikingly, while $merg1a$ expression was sustained at similar levels to embryonic development (Fig. 7A), $merg1b$ was dramatically up-regulated in adulthood, suggesting an important role for this isoform in heart homeostasis. $Nkx2-5$ levels were substantially decreased in normal adult hearts (Fig. 7A), and even further decreased in $Nkx2-5^{+/+}$ hearts, to approximately 50% of the homeostatic levels of WT controls (Fig. 7B), similar to the observed expression for heterozygous embryonic hearts (Fig. 2B). Notably, in line with the observed lack of ECG abnormalities in homeostasis, all analysed channels were not significantly changed in $Nkx2-5^{+/+}$ heterozygous adults (Fig. 7B), except for $merg1b$, which once again displayed significant changes with a dramatic increase in expression in $Nkx2-5^{+/+}$ hearts compared with controls. Unexpectedly, $Nkx2-5$ protein level in $Nkx2-5^{+/+}$ adult hearts was also similar to WT mice. Taken together, the data demonstrate a high degree of compensation of gene expression in adult heterozygous mice, supposedly important to keep normal homeostatic function.
DISCUSSION

This study describes a new mouse genetic model for the conditional or full deletion of Nkx2-5. Unlike other previously published models, our mouse line was specifically designed to fully remove all coding exons of the Nkx2-5 locus, ensuring that no residual protein is produced. We validated this line using a full knockout strategy and demonstrate anticipated commonalities with previously published knockout models (Lyons et al., 1995; Tanaka et al., 1999). In particular, we observed embryonic lethality around 10.5 dpc from heart morphogenetic impairment and down-regulation of Nppa and Gja5, both direct targets of Nkx2-5. We believe this line will be useful to unequivocally pinpoint aspects of Nkx2-5 function in different organs or sub-compartments of a particular organ.

The mechanisms governing the precise and organized chain of events responsible for correct electric propagation in the heart are extremely complex, requiring coordinated opening and closing of specific ion channels in a tightly controlled spatial and temporal manner (Bers, 2002). These events depend on interrelationships among diverse cardiac sub-regions that either generate the action potential (sinoatrial node, the primary pacemaker) or propagate electric activity to the ventricles (via atrioventricular node, His fibers and Purkinje network) and contractile cardiomyocytes present in all chambers (Bers, 2002). In humans, Nkx2-5 mutations are associated with conduction and electrophysiological defects, including atrioventricular block and ventricular arrhythmias (Clark et al., 2006; Perera et al., 2014; Schott et al., 1998). Nkx2-5 is detectable throughout the heart, from early embryonic stages to adult life. It is essential for specification of cardiomyocytes from mesodermal cells (Elliott et al., 2010) and the formation and homeostasis of atrioventricular node cells (Jay et al., 2004; Pashmforoush et al., 2004). Recently, Nkx2-5 has been implicated in the regulation of Scn5a (fast Na current, I_{Na}) and RyR2 (responsible for Ca^{2+} release from sarcoplasmic reticulum storage) expression (Briggs et al., 2008). Yet, not much is known about Nkx2-5 regulation of other ionic targets in the heart. A putative binding site for Nkx2-5 in the regulatory elements of the heart specific isoform of Cacna1c (type L_{Ca^{2+}} current) has been previously described but no evidence for direct regulation was established (Pang et al., 2003). Using our newly generated knockout line, we have dissected novel aspects of Nkx2-5 driven electrical regulation of heart ion channels. We have shown that Nkx2-5 is essential for the regulation of Cacna1c and that of the merg1b isoform of the Kcnh2/ERG channel (delayed repolarization channel, I_{Kr} current). In humans, mutations of KCNH2 cause the autosomal-dominant long-QT syndrome type 2 (LQT2), through the disruption of the normal function of I_{Kr} current. This disorder can lead to syncope and sudden death due to ventricular arrhythmias and fibrillation (Curran et al., 1995). merg1a is highly expressed in heart and brain (London et al., 1997), both tissues that are highly electrically active. merg1a encodes for a 1162
amino-acid protein, while *merg1b* lacks the first 59 amino acids, displays a divergent N-terminus and is less abundant (London et al., 1997). These two isoforms produce proteins that co-assemble to form channels with cardiac delayed rectifier potassium current $I_{Kr}$ properties (Erg mediated currents), and it is proposed that the relative levels of these isoforms might be essential for precise control of this current (London et al., 1997).

Using a bioinformatics search based approach, we identified putative distal (D1 and D2) and proximal (P) controlling regions upstream of the transcription start site of the isoform *merg1b* that contain several Nkx2-5 related binding sites (NKE). The proximal regulatory region displayed an open chromatin profile specifically in the heart, indicative of tissue specific interactions (Suppl. Fig. 2). Analysis of histone modifications showed that *merg1b* enhancer and promoter regions were enriched for active marks in cardiac tissue. The putative distal region lacked any of these markers, implying that this region is probably not active in the heart. Interestingly, both distal and proximal elements were shown to be capable of specific interaction with Nkx2-5 in HL-1 cells (Fig. 4C), but the distal element was not essential for *Merg1b* expression (Fig. 4D). Activation and function of cardiac promoters is highly dependent on the formation of stable transcriptional complexes with several cardiac specific and ubiquitous factors and co-factors (He et al., 2011; Linhares et al., 2004; Schlesinger et al., 2011; Sepulveda et al., 1998). Consistently, the *merg1b* proximal element had a NKE site juxtaposed to a putative Gata4 site and displayed synergistic activation by the complex Gata4/Tbx5, while either of these factors individually only had a low transcriptional effect (Fig. 4D). Combinatorial interactions of transcription factors competing for similar binding sites in regulatory regions have been shown to direct spatial compartmentalization within the heart (Habets et al., 2002; He et al., 2011). *merg1b* shows a highly dynamic temporal regulation, with increased levels in adults when compared to its expression in the embryonic heart (Fig. 7A).

Despite being directly activated by Nkx2-5, *merg1b* levels are paradoxically up-regulated in Nkx2-5<sup>+/−</sup> adult mice. Nkx2-5 is broadly expressed in the adult heart (Elliott et al., 2010) and has been shown to function as both repressor or activator within the heart field during embryonic development (Prall et al., 2007). The complex function and spatial distribution of Nkx2-5 may have relevant roles for *merg1b* spatial regulation. It remains to be seen if the combination of other events with heart-specific transcription factors can direct *merg1b* expression to specific compartments of the heart, as seen for expression of genes regulating atrioventricular canal specification (Habets et al., 2002). Furthermore, overexpression of the mutant Nkx2-5 I183M protein that has impaired transcriptional activity (Costa et al., 2013) causes a significant impairment of the $I_{Kr}$ current in HL-1 cardiomyocytes.
(Fig. 5B), known to express cardiac transcriptional factors, including Nkx2-5, structural proteins, receptors and ion channels (White et al., 2004). These cells express both Kcnh2 isoforms and possess I_{Kr} currents with highly similar properties to the native currents seen in primary cardiomyocytes (Toyoda et al., 2010). The decreased amplitude and maximum rate of rise (MRR) seen in Nkx2-5 I183M constructs also confirm the effect of the human mutation in the I_{Na} currents and must contribute to the overall conduction defects observed in patients with NKX2-5 mutations.

Our data shows that a 50% reduction of Nkx2-5 transcript level is compatible with sustainable cardiac activity under homeostasis (Fig. 6). We observed a moderate dilation and a high percentage of large PFOs in Nkx2-5^{Δ/+} hearts through morphological inspection, corroborated by reduced left ventricular function by MRI. Although not severe, these defects may contribute to disease phenotype under situations of physiological stress. By contrast no proper ASDs were identified, one of the most common defects detected in patients. These observations demonstrate that mouse models relying on the complete absence of one Nkx2-5 allele may not fully reproduce human disease, as most mutations identified in patients so far cause partial disruption of protein structure, as the previously described for I-M mutation (Costa et al., 2013). In spite of that, heterozygous and homozygous deletion models remain essential to allow the dissection of primary targets and pathways, because they can identify the direct effects caused by decrease levels of gene expression, as described in this work and several others.

No overall electrical conduction defects were observed in adult hearts under homeostatic conditions (Fig. 6C). Combined with the observed up-regulation of mrglb, these findings suggest that essential compensatory mechanisms take place to balance decreased levels of Nkx2-5, an event likely to be essential for survival to adulthood. Important targets may also be subjected to differential transcriptional regulation in adults, as demonstrated for the Nppa gene (Warren et al., 2011). A mild prolongation of the P-R interval was previously described using another model, where the first exon of Nkx2-5 was disrupted by insertion of a GFP reporter (Biben et al., 2000). In a third model, where the entire second exon was deleted, hypoplastic atrioventricular node and decreased amplitude of the His bundle electric signal were detected, followed by an age dependent prolonged P-R interval and shortening of QRS intervals (Jay et al., 2004). A more detailed dissection of the electrical activity in these mice showed the presence of mild abnormalities in ventricular conduction associated with decreased density of Purkinje fibers (Meysen et al., 2007). Furthermore, conditional deletion of Nkx2-5 in the atrial compartment led to hyperplastic sinoatrial node (Nakashima et al., 2014), while late embryonic and adult ventricular ablation led to hypoplastic atrioventricular node and progressive
atrioventricular block (Briggs et al., 2008; Pashmforoush et al., 2004; Takeda et al., 2009; Terada et al., 2011). In our model, we observed a high degree of transcriptional compensation in adult Nkx2-5Δ/+ animals, despite a persistent decrease in Nkx2-5 mRNA levels (Fig. 7B). Nkx2-5 protein stability is regulated by the Ubiquitin Proteasome System. Mutations in the homeodomain (DNA binding) region of Nkx2-5 lead to increased protein stability in vitro (Costa et al., 2013). It remains to be seen if change in protein stability in heterozygous mice can explain the compensation observed in this study. Our findings might also explain some divergence observed between our model and previously described Nkx2-5 ablation models, where only part of the Nkx2-5 gene is deleted, raising the importance of full characterization of the model to allow proper interpretation associated with the study of disease mechanisms.

All ion channels analysed here had comparable levels to adult WT mice, except for merg1b. This might explain the absence of electrical disturbances in adult hearts. Alternatively, previous works relied on invasive techniques and anesthetized/paced methodologies that can unmask electrical changes, while our recordings on non-anesthetized animals generate signal under homeostatic state. It is also possible that the different strategies for the engineering of the various Nkx2-5 mutant alleles may contribute to this divergence. Our new model completely abolishes Nkx2-5 expression through the deletion of both exons, while previous models either maintained part of the gene (Briggs et al., 2008) or inserted reporter or cre proteins in its locus (Biben et al., 2000; Prall et al., 2007).

Here we show that compensatory mechanisms occur in adults that bypass some of the effects seen in the hemi and homozygous models. For example, Nkx2-5 direct targets such as Nppa and several ion channels are expressed correctly in adult heterozygous hearts, but such compensation is not sufficient to sustain normal merg1b levels (Fig. 7B), thus possibly causing predisposition to electrical disturbances commonly seen in NKX2-5 patients and mouse models. Recently, a novel Nkx2-5 knockin mouse model has been described, where a mutation at the position R52G of the homedomain region led to high penetrant septal/valvular malformations and ventricular non-compaction (Ashraf et al., 2014). These malformations were highly dependent on the genetic background used and are much more severe then the phenotype in patients.

Collectively, our data demonstrate that a reduction in 50% of Nkx2-5 transcript levels is compatible with homeostatic heart function in mice, likely due to post-transcriptional mechanisms that counterbalance protein levels in Nkx2-5Δ/+ hearts.
Finally, using this novel line we also demonstrated that Nkx2-5 regulates several of the most important cardiac ion channels during embryonic heart development, including novel targets \textit{Cacna1c} and \textit{Kcnh2}, isoform \textit{merg1b}. These channels play essential roles in the proper generation and propagation of the action potential the heart. Given the broad distribution of Nkx2-5 protein in the heart, the current study provides a new model to fully remove \textit{Nkx2-5} from specific subcompartments and provides additional insights into the mechanisms of action of Nkx2-5 in the generation of electric and functional abnormalities in disease states. Our data also open new avenues for dissection of the complex etiology of NKX2-5 driven arrhythmias in patients, generating novel therapeutic targets for anti-arrhythmogenic therapies.
MATERIALS AND METHODS

Generation of mouse lines and genotyping
A novel conditional line for Nkx2-5 was achieved by generating a floxed allele (Nkx2-5cKo) encompassing both Exons 1 and 2, engineered by the Gene Recombineering and ES Cell Targeting Facilities of Monash University. A full knockout line (Nkx2-5Δ) was subsequently obtained by crossing Nkx2-5cKo mice with the CMV-cre deleter line (Schwenk et al., 1995). Genotyping was confirmed by PCR from genomic DNA prepared from tail biopsies using primers described in Supplementary Table 2. All mice were kept on a full C57BL/6J background and housed at Monash Animal Services. Mice were sacrificed by cervical dislocation or CO₂ inhalation. This investigation conforms with ethics application MARP-2011-175 (Monash University) and animal procedures were performed conform the Australian and NIH guidelines (Guide for the care and use of laboratory animals).

In situ hybridization
In situ hybridization methods were as described (Biben and Harvey, 1997). Probes were generated by RT-PCR and cloned into pGEMTeasy (Promega). To obtain antisense transcripts, reactions were performed using T7 RNA Polymerase (Roche).

Quantitative polymerase chain reaction
Total RNA was isolated using RNAqueous 4PCR kit (Invitrogen). cDNA synthesis was performed using the Superscript VILO kit (Invitrogen) following manufacturer’s instructions. qPCR reactions were performed using SYBR green master mix (Roche) and analysed using the LightCycler480 (Roche). At least 2 individual experiments in triplicate were performed. Primers are described in Supplementary Table 2.

Western blot and immunofluorescence
Protein was extracted from 15-week old adult mouse hearts using RIPA buffer and protease inhibitor cocktail (Roche) and quantified by Lowry method. 20µg of total protein was loaded in Any-Kd TGX gels (Bio-Rad) and transferred into Immobilon PVDF membrane (Millipore) for blotting. Proteins were detected using mouse Nkx2-5 (R&D) and mouse TnI RV-2 (DSHB) primary antibodies. TnI was used as loading control. Secondary antibodies were: anti-mouse IRDye 800 (LICOR) and anti-rabbit IRDye 680 (LICOR). Membranes were scanned using the Odyssey System (LICOR). For immunofluorescence experiments, E9.5 embryos were snap-frozen in OCT, after which 10µm sections were obtained using a cryostat (Leica). Primary antibodies were: anti-goat Nkx2-5 N-19 (Santa Cruz Biotechnologies), mouse TnI RV-2 (DSHB), rabbit Kcnh2 (Abcam). Secondary
antibodies were: anti goat AlexaFluor 550, anti mouse AlexaFluor 488, anti rabbit AlexaFluor 550. Nuclei were counterstained using DAPI.

**Magnetic Resonance Imaging (MRI) and Electrocardiogram (ECG)**

14-16 week-old adult mice were anaesthetised with 1.5-2.0% isoflurane for non-invasive imaging in a prone position before the surgical procedure. Imaging was performed in spontaneously breathing animals in a 9.4Tesla MR scanner (Agilent) to assess left (LV) and right ventricle (RV) chamber dimensions using T1 and T2-weighted anatomical cine-sequences. Measurements of cardiac function were performed using Segment (Heiberg et al., 2010). Electrocardiogram was obtained for non-anesthetized animals using the ECGenie system and intervals calculated using EzCG (Mouse Specifics). Statistics were determined by ANOVA and Student’s t-test.

**Atrial septal analysis**

To assess for the presence of patent foramen ovale (PFO) and atrial septal defect (ASD), adult mouse hearts were dissected and visually inspected under a stereoscope. Briefly, the left atrial appendage was removed to expose the atrial septum and record blood flow induced by pressurizing the right atrium, as previously described (Biben et al., 2000). Septa with normal morphology but a patent flap valve were scored as PFOs. Direct overlap of the ostium secundum and the foramen ovale was scored as an ASD. Excess or hyperelastic tissue over the foramen ovale, capable of protruding significantly when pressurising the right atrium, was scored as atrial aneurysm.

**Cell culture and transfection**

COS-7 cells were grown in DMEM (GIBCO) with 10% fetal bovine serum (FBS, GIBCO). HL-1 cardiomyocytes were grown in Claycomb media (Sigma) with 10% FBS and nonepinephrine (Sigma), maintained in a humidified 5% CO₂ incubator at 37°C. Cells were transfected using Lipofectamine/Plus (Invitrogen) or electroporated (NEON system, Invitrogen) following manufacturer’s instructions. Reporter constructs and plasmids containing Nkx2-5, Gata4 and Tbx5 were previously described (Linhares et al., 2004). For electrophysiological measurements, a control pmCherry (Clontech) vector was included. Kcnh2 promoter fragments were cloned by PCR into pGL3 basic vectors (Promega). Results were expressed as fold activity after normalization for Renilla activity.

**Chromatin Immunoprecipitation (ChIP)**

ChIP experiments were performed as previously described (Polo et al., 2010). NKE sites present in the Nppa proximal promoter region were used as a positive control (Linhares et al., 2004).
Immunoprecipitation was performed using a polyclonal antibody to Nkx2-5 H114–X (Santa Cruz). Enrichment of the NKE sites at different positions was validated by qPCR.

**Patch-clamp and calcium recordings**

Isolated transfected HL-1 cells were voltage-clamped using whole cell or nystatin-perforated patches (Axopatch200 and Digidata1322A, pClamp9) as described (Parkington et al., 2014; Toyoda et al., 2010). Briefly, under voltage-clamp, cells were step-depolarized from −50 mV and this activated outward currents. Biphasic currents upon repolarization to −50 mV were attributed to ERG currents. This current waveform arises from the very rapid recovery from inactivation, resulting in an increased ERG current that then decays as a tail current due to de-activation of the ERG channels. For these experiments patch electrode solution contained (mM): KCl 130, MgCl$_2$ 1.2, ATP 3, EGTA 5, HEPES 10. To enhance K$^+$ currents, the extracellular bathing solution contained (mM): KCl 140, MgCl$_2$ 1, HEPES 10, glucose 10, CaCl$_2$ 0.1, applied immediately before testing. In other experiments, current-clamp mode was used to record action potentials. The ERG blocker dofetilide stock in dimethylsulphoxide (DMSO, Sigma) was diluted 1:10,000.

**Acknowledgments**

We acknowledge the use of Monash University MicroImaging and Biomedical Imaging facilities.

**Sources of Funding**

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**Disclosures**

None.
References


Figures legends

**Figure 1. Schematics showing Nkx2-5 mouse locus and engineered constructs.**

(A) Endogenous Nkx2-5 mouse locus with 5’ and 3’ untranslated regions (UTRs) in white boxes and coding exons in black boxes. Probes used for Southern blot are shown (5’P and 3’P). (B) Targeting vector showing position of loxP sites (black arrowheads) and PGK-Neo selection cassette inserted on 3’ UTR, flanked by FRT sites (gray). (C-D) Targeted conditional Nkx2-5 locus following homologous recombination and deletion of FLP-Neo cassette (cKo line). (E) Nkx2-5 locus after conditional deletion using ubiquitously expressed CMV-cre (delta or null locus). (F) Validation of targeting using Southern blot on selected stem cell clones to detect wild-type (wt) and conditional alleles (cKo) and (G) genotyping strategy used to detect wt (+/+), heterozygous (∆/+ and homozygous (∆/∆) alleles following deletion with CMV-cre recombinase.

Bg-BglI; E-EcoRV; M-MluI; Nc-NcoI; N-NotI; P-PvuII; X-XbaI; Xh-XhoI.

**Figure 2. Characterization of the embryonic phenotype of Nkx2-5 null embryos.**

(A) Left sided whole mount view of Nkx2-5δ/δ homozygous embryos, showing overall reduced size and hypoplastic unlooped hearts at 9.5, ultimately resulting in lethality by 10.5dpc (N=20). (B) Nkx2-5 message levels detected by qPCR show around 50% reduction in Nkx2-5∆/+ animals and are virtually undetectable in Nkx2-5δ/δ homozygous embryos. (C) Immunofluorescence for Nkx2-5 in 10.5 dpc embryos shows complete absence of protein in Nkx2-5δ/δ embryos. Red – Nkx2-5; Blue – DAPI. (D) In situ hybridization reveals dramatic reduction of expression of Nkx2-5 direct targets Nppa (atrial natriuretic factor, N=3) and Gja5 (connexin 40, N=3) in the heart of Nkx2-5δ/δ homozygous mutant embryos, as well as in the systemic vasculature for Gja5. Expression of the indirect Nkx2-5 target Mlc2v was mostly normal in all embryos analysed (N=3). T test was used to determine statistical significance; ***p<0.001, ****p<0.0001.

**Figure 3. Nkx2-5 controls cardiac ion channel levels during embryonic development.**

qPCR analysis of several cardiac ion channels in WT, Nkx2-5δ/+ and Nkx2-5δ/δ embryos (N=3) at 9.5 dpc shows a significant decrease in levels of voltage activated fast Na⁺ channel Scn5a (A), voltage activated L-type Ca²⁺ channel Cacna1c (B) and the voltage activated delayed K⁺ rectifier channel Kcnh2 (C), but no significant changes in the Ca²⁺ reticulum ATPase Serca2 (D). T test; *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.
Figure 4. Nkx2-5 binds to and directly regulates the Kcnh2 isoform merg1b.

(A) qPCR for Kcnh2 isoforms merg1a and merg1b shows that Nkx2-5 regulates both isoforms, although a more robust response is observed for merg1b expression. (B) Bioinformatic analyses of the genomic region of Kcnh2 showed the presence of putative regulatory regions upstream of the transcriptional start site of the Kcnh2 isoform merg1b (black boxes proximal and distal, encased by grey areas aligning with chromatin marks). Regulatory regions have enriched methylation markers (red peaks). merg1a 5’ regulatory regions are not shown in this drawing. (C) ChIP in HL-1 cells shows direct binding of endogenous Nkx2-5 to 2 sites (P and D2) in the upstream region of merg1b transcriptional start site. A previously identified Nppa element with 2 NKE binding sites was used as a positive control. (D) P element (NKE/G4 – grey box) in upstream region of merg1b is synergistically regulated by cardiac transcription factors Nkx2-5, Gata4 and Tbx5 in COS-7 cells using over-expression constructs. Absence of distal element (D1+D2 – red box) has no effect on transcriptional activity. (E) Mutagenesis of NKE site P (left panel) leads to a significant decrease in transcriptional activity of merg1b in HL-1 cells (right panel), showing that this is site is the major element regulating merg1b expression when endogenous transcription factors are used to measure luciferase activity. Right panel from top to bottom: black bar – promoter-less luciferase activity; red bar – full construct containing P and D elements; red bar with black pattern – deletion of D element; red bar with white squares – full construct containing mutated P element; blue bar – truncated construct with (1087 bp) with intact P element; blue bar with white squares – truncated construct with mutated P site. T test; ** p<0.01, ***p<0.001, ****p<0.0001.

Figure 5. Transcriptional impairment of Nkx2-5 leads to decreased activity of I_{kr} current (ERG) and calcium handling in HL-1 cells.

(A) Cells successfully transfected were clamped following identification of mCherry fluorescence from reporter construct (arrows). (B) Family of endogenous membrane ERG currents in HL-1 cells transfected with empty vector (left middle panel) and disappearance of currents after addition of ERG inhibitor (left bottom panel). Right panels depict ERG currents for transfected Nkx2-5 I183M mutant protein, respectively. Top panels: voltage clamp protocol (N=10; T test, *p<0.05). (C) Patch-clamp analysis of ERG channel activity shows decreased currents in HL-1 cells expressing the mutant Nkx2-5 I183M protein when compared with control cells (vector). (D-E) Whole cell action potential of transfected HL-1 cells and analysis of Peak Amplitude and Maximum Rate of Rise (MRR) (N=10 independent cells; T test, ***p<0.001, ****p<0.0001).
Figure 6. *Nkx2-5Δ/+* mice develop moderate left ventricular dysfunction and show no overall changes in electric activity in homeostasis. (A) Whole mount (frontal view – left) and trichrome stained transverse sections of WT and *Nkx2-5Δ/+* hearts, showing mildly dysmorphic chambers in heterozygous *Nkx2-5Δ/+* hearts (N=10). (B) MRI measurements for left ventricular ejection fraction (LV-EF), myocardial mass (LV-mass), cardiac output (LV-CO), end diastolic volume (LV-EDV), end systolic volume (LV-ESV) and stroke volume (LV-SV) are shown. While all parameters showed a trend for worsening of function in *Nkx2-5Δ/+* hearts, only left ventricle mass and stroke volume were significantly reduced (N=6, p<0.05). (C-D) Representative ECG tracings of WT and heterozygous animals. Cardiac electrical intervals PQ, QRS and QT were not affected in non-anesthetized *Nkx2-5Δ/+* animals (WT, N=12; *Nkx2-5Δ/+*, N=8).

Figure 7. Cardiac ion channels show dynamic temporal regulation and a high degree of compensation at the transcriptional level in *Nkx2-5Δ/+* adult hearts. (A) qPCR analysis of *Kcnh2* isoforms shows that relative levels of *merg1a* are maintained between 9.5 dpc embryos and adulthood. *merg1b* levels are increased 4 fold in adult hearts, while *Nkx2-5* levels are decreased by 2 fold. (N=3) (B) Message for ion channels show no difference in levels in *Nkx2-5Δ/+* adult hearts when compared to WT mice, except for *Kcnh2* isoform *merg1b*, which shows a 3 fold increase in expression level (N=3). T test, *p<0.05. (C) Protein levels for Nkx2-5 were not changed in *Nkx2-5Δ/+* adult hearts when compared to WT hearts (N=3 each).
Supplementary Table 1 – Functional analysis of adult Nkx2-5<sup>−/−</sup> and control animals

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*p<0.05
### Supplementary Table 2. Primers used

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Genotyping

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Cloning Kcnh2 promoter region

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ChIP

| Kcnh2 NKE 1 F | TGTCCGCAAGAGTATGATTC |
| Kcnh2 NKE 1 R | GTCGATTTTGGTACACCCGC |
| Kcnh2 NKE 2 F | GGGTGATACAAAAGAAGCTG |
| Kcnh2 NKE 2 R | CAACCGGTGCTGGTGGG |
| Kcnh2 NKE 3 F | CTGCACTGTTGAGGGAGT |
| Kcnh2 NKE 3 R | ACTGGCATTATGGGCTG |
| ms-chip_Snail2 F | TGTGATGAGGGAGT |
| ms-chip_Snail2 R | AAGTGGGGCTGTCCAG |
| pANF ChIP_For | CCCAGGAAGATAACAGAGGACG |
| pANF ChIP_Rev | CACATTCTTGCTGATTTGGGC |

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Supplementary Figures

**Supplementary Figure 1. Generation of the conditional (cKo) line**

(A-C) To obtain the cKo line the original line containing the flp—flaked Neomycin (Neo) cassette was crossed with the line Cg-Tg (ACTFLPe) 9205Dym/J (FlpE - Jackson Laboratory) that expresses the FLP1 recombinase under the control of the ACTB promoter. Location of primers used for selection of correctly recombined mice are shown. (D) PCR showing successful deletion of the Neo cassette by FLP1 recombinase in lane 1. (E) PCR genotyping of heterozygous Nkx2-5<sup>cKo/+</sup> (lane 1) and WT littermates (lanes 2-4). WT band: 398bp; 2-5flox (with Neo): 327bp; FLP-2-5flox (without Neo): 521bp.

**Supplementary Figure 2. Nkx2-5 mutant embryos show similar levels of ERG protein.**

Immunofluorescence of E9.5 hearts shows ERG staining in red and troponin I (TnI) in green. Similar levels of ERG are seen in WT, heterozygous and homozygous mutant hearts.

**Supplementary Figure 3. Bioinformatics analysis of conserved tissue-specific putative regulatory regions on the Kcnh2 locus.**

Global tissue analyses of putative regulatory regions on the Kcnh2 locus, showing that isoform merg1a regulatory region contains markers of active chromatin in the heart and other tissues while merg1b regulatory region markers are highly enriched to heart (red) and neural (gray) tissues only. Grey boxes mark proximal and distal putative Nkx2-5 binding regions identified in the merg1b upstream regions.
Highlights

- A new Nkx2-5 conditional knockout mouse that fully deletes the gene was engineered.
- Adult Nkx2-5 heterozygous mice show mild morphological and functional dysfunction.
- Nkx2-5 regulates ion channels, including Kcnh2 isoform merg1b.
- Nkx2-5 mutations lead to decreased ERG (Kcnh2) channel activity.
- Nkx2-5 regulates genes associated with electric propagation in the heart.
Figure 1

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A

Nkx2-5-locus

B

Nkx2-5-targeting vector

C

Nkx2-5-targeted locus

D

Nkx2-5-Neo deleted locus

E

Nkx2-5-delta locus

F

WT cKo

kb

PvuII (5’probe)

9.9

8.8

WT cKo

kb

EcoRV (3’probe)

41.1

6.9

9.9

8.8

1Kb

G

bp

WT cKo

PGK-Neo

Δ/+  Δ/Δ

621

398

Δ/+  Δ/Δ
Figure 3

Panel A: Scn5a

Panel B: Cacna1C

Panel C: Kcnh2

Panel D: Serca

**Figure 3**
Figure 4

Kcnh2 - merg1a

Kcnh2 - merg1b

Figure 4

Kcnh2 genomic locus, Mouse (mm9), chr5:23,824,498-23,839,509

Conservation profiles
Cardiac expression (RNA-seq)
Gene structure
Cardiac open chromatin profiles (DHS)
Heart-specific enhancers
Kcnh2 (merg1b), Kcnh2 (merg1a)

Figure 4
Figure 5

A

B

C

D

E

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Figure 6

A. Images of WT and Nkx2-5 Δ/+ hearts with scale bars indicating 1 mm.

B. Graphs showing comparison of LV parameters:
- LV-EF (%)
- LV-mass (mg)
- LV-CO (ml/min)
- LV-EDV (μl)
- LV-ESV (μl)
- LV-SV (μl)

C. Heart rate tracings for WT and Nkx2-5 Δ/+ mice.

D. Summary of QRS and QT parameters:
- QRS (ms)
- QT (ms)

**n.s.** indicates no significant difference.
Figure 7

Panel A: Quantitative real-time PCR analysis showing fold expression of Kcnh2-merg1a, Kcnh2-merg1b, and Nkx2-5 in E (left) and A (right) hearts.

Panel B: Comparison of fold expression between WT and Nkx2-5Δ/+ for Nkx2-5, Scn5a (Nav1.5), Cacna1C (Cav1.2), RyR2, and Kcnh2-merg1b.

Panel C: Gel electrophoresis images showing TnI expression in WT and Nkx2-5Δ/+.
Supplementary Figure 1

A. Nkx2-5-locus

B. Nkx2-5-targeted locus

C. Nkx2-5-Neo deleted locus

FP1 - located at CDS exon2
FP2 - located at PGK promoter
RP - located at 3'-UTR

D. [Image of gel electrophoresis]

E. [Image of gel electrophoresis]

D. [Image of gel electrophoresis]
Supplementary Figure 3

Kcnh2 genomic locus, Mouse (mm9), chr5:23,817,403-23,865,426

Gene structure

Kcnh2 genomic locus, Mouse (mm9), chr5:23,817,403-23,865,426

Conservation profile

Cardiac expression (RNA-seq)

Cardiac specific expression (RNA-sseq)

Heart, Spleen and Kidney

Liver, Cortex and Thymus

Bone Marrow

Small Intestine

Olfactory Bulb

Placenta

UCSC Genes

Supplementary Figure 3

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