Transcription factor ETV1 is essential for rapid conduction in the heart

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Rapid impulse propagation in the heart is a defining property of pectinated atrial myocardium (PAM) and the ventricular conduction system (VCS) and is essential for maintaining normal cardiac rhythm and optimal cardiac output. Conduction defects in these tissues produce a disproportionate burden of arrhythmic disease and are major predictors of mortality in heart failure patients. Despite the clinical importance, little is known about the gene regulatory network that dictates the fast conduction phenotype. Here, we have used signal transduction and transcriptional profiling screens to identify a genetic pathway that converges on the NRG1-responsive transcription factor ETV1 as a critical regulator of fast conduction physiology for PAM and VCS cardiomyocytes. Etv1 was highly expressed in murine PAM and VCS cardiomyocytes, where it regulates expression of Nkx2-5, Gja5, and Scn5a, key cardiac genes required for rapid conduction. Mice deficient in Etv1 exhibited marked cardiac conduction defects coupled with developmental abnormalities of the VCS. Loss of Etv1 resulted in a complete disruption of the normal sodium current heterogeneity that exists between atrial, VCS, and ventricular myocytes. Lastly, a phenome-wide association study identified a link between ETV1 and bundle branch block and heart block in humans. Together, these results identify ETV1 as a critical factor in determining fast conduction physiology in the heart.

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Rapid impulse propagation in the heart is a defining property of pectinated atrial myocardium (PAM) and the ventricular conduction system (VCS) and is essential for maintaining normal cardiac rhythm and optimal cardiac output. Conduction defects in these tissues produce a disproportionate burden of arrhythmic disease and are major predictors of mortality in heart failure patients. Despite the significant clinical impact, little is known about the gene regulatory network that dictates the fast conduction phenotype. Here, we have used signal transduction and transcriptional profiling screens to identify a genetic pathway that converges on the NRG1-responsive transcription factor ETV1 as a critical regulator of fast conduction physiology for PAM and VCS cardiomyocytes. Etv1 was highly expressed in murine PAM and VCS cardiomyocytes, where it regulates expression of Nkx2-5, Gja5, and Scn5a, key cardiac genes required for rapid conduction. Mice deficient in Etv1 exhibited marked cardiac conduction defects coupled with developmental abnormalities of the VCS. Loss of Etv1 resulted in a complete disruption of the normal sodium current heterogeneity that exists between atrial, VCS, and ventricular myocytes. Lastly, a phenome-wide association study identified a link between ETV1 and bundle branch block and heart block in humans. Together, these results identify ETV1 as a critical factor in determining fast conduction physiology in the heart.

Introduction

Heritable and acquired syndromes affecting fast conduction in the atria and ventricular conduction system (VCS) produce a broad spectrum of arrhythmic disease, including atrial fibrillation, ventricular tachyrhythmias, and heart block. In addition, aberrant VCS impulse propagation increases morbidity and mortality in heart failure patients because of electrical dyssynchrony (1). Despite the significant clinical impact of fast conduction disorders on the health care system, limited therapeutic options exist. Through an increased understanding of the molecular determinants of fast conduction, targeted therapies aimed at improving conduction parameters in the atria or VCS can be developed.

A hierarchy of myocardial conduction velocities exists within the heart to ensure optimal cardiac output. Fast conduction is an essential feature of the pectinated atrial myocardium (PAM) and VCS to coordinate and synchronize contraction of the cardiac chambers. The VCS is composed of Purkinje cells and is structurally divided into the His bundle, bundle branches, and the Purkinje fiber network (also collectively referred to as the His-Purkinje system). The specialized conduction properties of PAM and Purkinje myocytes are due to the enriched expression of key conduction genes, which include Scn5a (encoding the α subunit of the cardiac sodium channel Na,v1.5) (2) and Gja5 (encoding the high-conductance gap junction protein connexin40, or Cx40) (3). This unique expression profile imparts to these cell types distinct electrophysiological features, including cell type–specific biophysical properties of the sodium current (4, 5) and rapid impulse propagation between neighboring cardiomyocytes (6, 7).

Common to all rapidly conducting tissues is their origins within the pectinated and trabeculated myocardium during atrial and ventricular chamber formation, respectively. During this stage, pectinated and trabecular myocytes grow as sheet-like layers along the endocardial surface of the cardiac chambers and acquire the fast conduction gene program, which is maintained into adulthood in the PAM and VCS. How these pectinated/trabecular myocytes acquire and maintain the fast conduction gene program is incompletely understood. Two transcription factors, the homeobox factor NKX2-5 and the T-box factor TBX5, are both known to work cooperatively in specification of the VCS (8–10). Mutations in NKX2-5 or TBX5 result in congenital heart disease and conduction defects, such as atioventricular (AV) block and/or bundle branch block in humans (11–15) and mouse models (10, 16–18). Yet what remains unresolved is how these factors, which are broadly expressed in the heart, contribute to the fast conduction phenotype only within pectinated and trabeculated myocytes that ultimately become the PAM and VCS, respectively. Moreover, a perinatal knockout of Nkx2-5 did not show an appreciable change in Na,v1.5 expression in the atria and VCS (19). Therefore, we hypothesized that additional cell type–specific transcriptional regulators must be playing a role in activating the fast conduction gene program exclusively within the PAM and VCS.

To identify novel transcriptional regulators of the fast conduction phenotype, we made use of our previous observation that neuregulin-1 (NRG1) is sufficient to upregulate cardiac conduction system–lacZ (CCS-lacZ) reporter gene expression throughout...
embryonic hearts in culture (20, 21). Based on this observation, we performed a 2-component screen using a signal transduction inhibitor assay in CCS-lacZ hearts in tandem with gene profiling of developing and mature Purkinje cells. Through this dual screen, a single candidate, Etv1, a member of the E-twenty-six (ETS) transcription factor family, was identified. ETVI is a NRGI-responsive factor that is highly expressed in PAM and VCS myocytes, where it establishes the fast conduction phenotype through enrichment of Nkx2-5, Gja5, and Scn5a. Consequently, Etv1-deficient mice exhibit cardiac conduction defects and developmental abnormalities of the VCS. Etv1 KO hearts display a loss of the normal sodium current heterogeneity that exists between Purkinje, atrial, and ventricular myocytes. A phenome-wide association study (PheWAS) (22) identified associations between ETVI and bundle branch block and heart block in humans, suggesting conservation of this ETVI-dependent pathway in mice and humans.

Results

NRGI upregulates CCS-lacZ reporter gene expression in embryonic hearts through an RSK/MSK-dependent signaling pathway. We previously showed that NRGI treatment of embryonic day 9.5 (E9.5) CCS-lacZ hearts in culture was able to significantly increase

Figure 1. NRGI regulates CCS-lacZ gene enrichment through MAPK signaling. (A) Representative E9.5 X-gal–stained CCS-lacZ hearts after in vitro culture with vehicle control or NRGI for 24 or 48 hours. OFT, outflow tract; V, ventricle; A, atria. (B) Immunofluorescence staining of E9.5 hearts cultured with vehicle control or NRGI for 24 hours for expression of NKKX2-5, Cx40, Na1.5, and IRX3. (C) Schematic representation of NRGI-ErbB2/ErbB4 intracellular signaling highlighting pathway-specific kinase inhibitors (red). (D) Representative E9.5 X-gal–stained CCS-lacZ hearts cultured with vehicle control or kinase inhibitors in the presence of NRGI. Top row: Representative images presented in A. (E) Quantification of CCS-lacZ expression, determined as a ratio of X-gal–positive area to the total heart area (n = 4). (F) Quantitative RT-PCR of Nkkx2-5, Gja5, Scn5a, and Irx3 from E9.5 hearts in culture treated with vehicle control, NRGI, or NRGI plus PD98059 for 24 hours. Red boxed region in A corresponds to location of immunofluorescence image in B. Nuclei were stained with DAPI (blue, B). NRGI concentration for all experiments was 2.5 × 10−9 M. Doses used for kinase inhibitor studies were as follows: PP2 (10 μM), LY294002 (25 μM), PD98059 (50 μM), FR18204 (20 μM), SL0101 (50 μM), H89 (10 μM). Scale bars: 200 μm (A and D); 50 μm (B). Data represent mean ± SEM. *P < 0.05, 1-way ANOVA.
reporter gene expression (20). To confirm that NRG1 treatment upregulates bona fide fast conduction genes and not just CCS-lacZ expression (Figure 1A), we immunostained treated hearts for NKX2-5, Cx40, Na1.5, and Iroquois-related homeobox 3 (IRX3) (ref. 23 and Figure 1B). NRG1 treatment enriched expression of these gene products throughout the heart. Next, we sought to identify the critical NRG1-dependent signal transduction pathway that mediates CCS-lacZ and fast conduction gene enrichment using a kinase inhibitor strategy.

Binding of NRG1 to its cognate receptor, ErbB4, results in heterodimerization with ErbB2, which activates numerous downstream signal transduction events, including Src, PI3K, and fast conduction gene enrichment (24–27; and schematic, Figure 1C). Selective inhibition of Src or PI3K signaling did not perturb downstream signal transduction events, including Src, PI3K, and Ras-MAPK pathways (refs. 24–27; and schematic, Figure 1C). Selective inhibition of Src or PI3K signaling did not perturb NRG1-dependent CCS-lacZ upregulation (Figure 1, D and E). In contrast, inhibition of MEK in the Ras-MAPK pathway completely abrogated CCS-lacZ enrichment in response to NRG1 treatment (Figure 1, D and E). MEK inhibition also reduced basal levels of CCS-lacZ expression (Figure 1E and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI87968DS1). Inhibition of downstream targets of MEK, which include ERK1 and ERK2, 90-kDa ribosomal S6 kinase (RSK), and mitogen- and stress-activated protein kinase (MSK), resulted in similar blockade of NRG1-mediated CCS-lacZ upregulation (Figure 1, D and E, and ref. 28). Confirming the CCS-lacZ reporter screen, NRG1 treatment of E9.5 hearts significantly increased transcript levels of Nkx2-5, Gja5, Scn5a, and Irx3 (Figure 1F), while cotreatment with NRG1 plus MEK inhibitor (PD98059) completely blocked fast conduction gene upregulation.

The NRGI- and MAPK-responsive transcription factor Etv1 is highly enriched in fast conduction tissues. In parallel with the signal transduction screen, we generated differential gene expression libraries at multiple developmental time points (E10.5, E12.5, and postnatal day 56 [P56]; ref. 29) to identify Purkinje-enriched transcription factors (Figure 2A). For embryonic stages, trabecular myocytes, from which VCS cells derive, were separated from compact ventricular myocytes using laser capture microdissection. For adult stages, we used a dual reporter system to identify cardiomyocytes (Myh6-Cre LSL-ttdTomato) and cardiac conduction system cells (Cntn22EGFP/−) (29, 30). Myh6-Cre LSL-ttdTomato Cntn22EGFP/− hearts were enzymatically dissociated to collect ventricular myocytes (identified as Tomato+/CNTn2EGFP−) and Purkinje cells (identified as Tomato+/CNTn2EGFP−) by flow cytometry (29). Expression libraries were then generated using the Affymetrix gene array platform. Analysis of our gene lists for VCS-enriched transcription factors yielded 8 candidate factors present at all developmental time points (Figure 2A). Etv1 was the most highly enriched transcription factor in adult Purkinje cells (Figure 2B). Etv1 enrichment in Purkinje cells was confirmed by quantitative RT-PCR (qPCR) (Figure 2C). Interestingly, Etv1 is a known target of ErbB2 (31–34) and Ras-MAPK-RSK/MSK signaling pathways (34–36) and was therefore an attractive candidate.

Etv1 belongs to the PEA3 group of ETS family transcription factors, which are involved in cell fate decisions and in functional modulation of neuronal cell types (37–39). ETS family members regulate transcription through the ETS-DNA binding domain that recognizes a central 5′-GGAA/T-3′ motif (40). Using an unbiased approach, we performed transcription factor motif analysis on the promoters of the most highly enriched genes in the Purkinje data set, restricting our analysis to 1,000 bp upstream of the transcriptional start sites. The generalized ETS-binding motif (−log P = 25.0) and the ETV1-binding motif (−log P = 24.0) were the second and fourth most significantly enriched motifs in the Purkinje-enriched gene set (Figure 2D).
Etv1 expression is NRG1 responsive in embryonic and postnatal hearts. As NRG1 treatment can expand CCS-lacZ expression transmurally in embryonic hearts, we tested whether NRG1 would have a similar effect on Etv1 expression. Treatment of E9.5 Etv1nlz/+ hearts with NRG1 expanded reporter gene expression from an
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(Figure 5B) for activated components of the NRG1-ErbB2/4-MAPK signaling cascade. At E13.5, NRG1 expression was restricted to endocardial cells that were in direct contact with pectinated and trabecular myocytes. Phosphorylated (activated) forms of ErbB4 (pErbB4), ErbB2 (pErbB2), and ERK1/2 (pERK1/2) were restricted to PAM and ventricular trabecular myocytes, mirroring the expression patterns of ETV1 and Cx40 in serial sections (Figure 5A). At P21, NRG1 + (red) cells are interdigitated among PAM and CNTN2 + (green) Purkinje cells (Figure 5B). In the proximal and distal VCS, NRG1 + cells can be seen making numerous connections with CNTN2 + Purkinje cells (Figure 5B). Correspondingly, the PAM and CNTN2 + Purkinje cells are highly enriched in activated pErBb4 (Figure 5C). Phosphorylated (activated) forms of ERK1/2 and RSK were also enriched in PAM and Purkinje cells in P21 hearts (Supplemental Figure 3). To test dynamics of ETV1 activation, we treated dissociated neonatal rat atrial cardiomyocytes with NRG1, which resulted in serine phosphorylation (Figure 5D) and nuclear accumulation (Figure 5E) of ETV1.

Mice deficient in Etv1 exhibit cardiac conduction defects. To investigate whether ETV1 is an important regulator of cardiac conduction physiology, we performed electrophysiological analysis on Etv1nlz/nlz mice, herein referred to as Etv1 knockout (KO) mice. As Etv1 KO mice die uniformly at 3 weeks of age due to severe neuromuscular impairment, ECG analysis was per-
formed at P18 (38). At this age, Etv1 KO mice were smaller than their WT and heterozygous (Het) littermates but appeared relatively normal except for the previously described neuromuscular phenotype (38). Like AG825-treated mice, Etv1-deficient mice displayed cardiac conduction abnormalities as demonstrated by lengthening of the P wave, PR interval, and QRS wave durations (Figure 6A and Supplemental Table 1). In addition, 30% of Etv1 KO mice displayed bundle branch block (Figure 6A). Bundle branch blocks were never observed in WT or Het animals. Prolongation of the PR interval prompted further analysis of AV conduction using intracardiac electrogram recordings, which demonstrated prolonged atrial-His (AH) and His-ventricular (HV) intervals in Etv1 KO mice (Figure 6B and Supplemental Table 1). The AH interval is a surrogate measure of AV nodal conduction time, and the HV interval is a measure of VCS-dependent ventricular activation time. Etv1 KO mice had normal cardiac function as assessed by transthoracic echocardiography (Supplemental Table 2). Although Etv1 KO hearts were significantly smaller than their WT and Het counterparts, the heart weight-to-body weight ratios remained equivalent to those of control animals (Supplemental Figure 4A). There was no evidence of structural abnormalities or fibrosis in Etv1 KO hearts based on H&E (Supplemental Figure 4B) or trichrome staining (Supplemental Figure 4, C and D). In addition, Etv1 KO hearts did not display abnormalities of cell cycling (Supplemental Figure 5) or increased apoptosis by TUNEL staining (data not shown).

Etv1-deficient mice have reduced levels of Nkx2-5, Gja5, and Scn5a in the atria and VCS. Conduction abnormalities in Etv1 KO mice were evaluated using immunofluorescence staining to detect altered expression of fast conduction genes. The normal

Figure 5. Activated NRG1 signaling is restricted to regions of fast conduction and results in activation of ETV1. (A) Immunofluorescence staining of NRG1, pErB4, pErB2, pERK1/2, ETV1, and Cx40 in E13.5 embryonic heart serial sections. (B and C) Immunofluorescence staining of NRG1 (B) and pErB4 (C) in P21 atria and the His-Purkinje system. (D and E) Cultured neonatal rat atrial myocytes treated with vehicle control or NRG1 for 0, 1, or 3 hours were evaluated for ETV1 phosphorylation and nuclear accumulation. (D) ETV1 was immunoprecipitated from cell lysates followed by Western blot analysis to detect phospho-serine residues (p-Ser). Densitometry quantification of p-Ser levels (normalized to ETV1) presented relative to baseline (n = 4). (E) Nuclear accumulation of ETV1 with NRG1 treatment was detected using immunofluorescence staining. Percentage of ETV1 nuclear-positive area presented with respect to DAPI-positive area (dashed circles) (n = 20 cells). Nuclei were stained with DAPI; atrial myocytes were identified by α-actinin staining (blue, DAPI; red, ETV1; green, actinin). LA, left atria; LV, left ventricle. Data represent mean ± SEM. *P < 0.05, 2-tailed Student’s t test. Scale bars: 50 μm (A); 25 μm (B and C); 5 μm (E).
enrichment of NKX2-5, Cx40, and NaV1.5 in the atria, proximal VCS, and distal Purkinje cells was disrupted in P18 Etv1 KO hearts (Figure 7, A–C, and Supplemental Figure 6). NKX2-5 expression and NaV1.5 expression in the atria and VCS were reduced to ventricular levels. Similar reductions in NKX2-5 and NaV1.5 expression levels in atria and trabecular myocytes were evident in E13.5 Etv1 KO hearts (Supplemental Figure 7). In contrast, IRX3 levels remained unchanged in Etv1-deficient P18 hearts (Supplemental Figure 8). Western blot analysis of atrial samples from Etv1 KO mice demonstrated significantly reduced levels of NKX2-5, Cx40, and NaV1.5 compared with WT and Het controls (Figure 8, A–C, and Supplemental Figure 9). Western blot analysis of atrial samples from Etv1 KO mice showed significantly prolonged P, PR, and QRS intervals with respect to WT mice (n = 10). A subset of Etv1 KO mice (30%) displayed an Rs’ pattern.

In contrast, Etv1 KO atrial and Purkinje myocytes were significantly reduced in mutant atrial and Purkinje myocytes (Figure 7F and Supplemental Figure 4). Similar to the immunostaining results, Nkx2-5 and Scn5a RNA levels from Etv1 KO atrial and Purkinje myocytes were reduced to ventricular levels (Supplemental Figure 9). Interestingly, Tbx5 expression was significantly increased in mutant atrial and Purkinje myocytes, presumably through a compensatory mechanism (Figure 7F and Supplemental Figure 4). We next treated E9.5 Etv1 WT and KO hearts in culture with NRG1 and measured transcript levels of fast conduction genes. With NRG1 treatment, Etv1 KO hearts had a significantly blunted response of Nkx2-5, Gja5, and Scn5a RNA upregulation compared with WT (Figure 7G).

Etv1-deficient mice exhibit hypoplasia of the VCS due to a reduction in terminal Purkinje cells. Given the reduced levels of NKX2-5 in the VCS of Etv1 KO hearts, a detailed structural evaluation of the His-Purkinje system was performed. In humans, NKX2-5 mutations cause nonsyndromic congenital heart defects and AV conduction abnormalities (14). Mouse models of Nkx2-5 haploinsufficiency phenocopy the human condition and manifest prolonged PR and QRS durations on ECG as well as hypoplasia of the VCS with approximately 50% loss of terminal Purkinje cells (17, 18, 44, 45). To identify morphological defects in the conduction system, Etv1 KO mice backcrossed into the Cntn2EGFP+ background were studied. The left and right VCS of Etv1 KO hearts was hypomorphic, recapitulating the defects seen in Nkx2-5 haploinsufficient mice (Figure 8A, left and middle panels, and Supplemental Figure 10). Regional analysis of the left VCS (Figure 8B) demonstrated significant loss of terminal Purkinje cells, while the fascicles appeared less affected. X-gal staining of Etv1KO/+/mice confirmed the absence of terminal Purkinje cells (Figure 8A, right panels).

To quantify the percentage of CNTN2EGFP+ Purkinje cells in WT and KO hearts, ventricles were dissociated into single cells and stained with TMRM to identify cardiomyocytes (43). The percentage of TMRM+ CNTN2EGFP+ Purkinje cells relative to total ventricular myocytes was reduced by 52% in Etv1 KO hearts as assessed by flow cytometry (Figure 8C).

Etv1 KO hearts display loss of sodium current heterogeneity between Purkinje, atrial, and ventricular myocytes. Based on the conduction abnormalities in Etv1 KO mice and the reductions in Scn5a expression in atrial and Purkinje cells, we next studied the biophysical properties of the sodium current (I\textsubscript{Na}) in dissociated myocytes. The fast, voltage-gated I\textsubscript{Na} is a major determinant of myocardial excitability and conduction velocity. The biophysical properties of the I\textsubscript{Na} are not homogeneous between the atrial, Purkinje, and ventricular myocytes (4, 5). Atrial (4) and Purkinje (5) myocytes have greater maximum sodium conductance and correspondingly higher levels of Na\textsubscript{1.5} expression (2, 19). To verify and explore in greater detail the baseline I\textsubscript{Na} heterogeneity that exists between these major cardiac cell types, we applied whole-cell patch clamp technique to myocytes dissociated from Cntn2EGFP+ P18 hearts. The maximum conductance of I\textsubscript{Na} was significly different between all 3 cell types, with Purkinje (CNTN2EGFP+) cells having the greatest maximum conductance followed by atrial then ventricular myocytes (1.11 ± 0.09 nS/pF, 0.78 ± 0.03 nS/pF, 0.61 ± 0.03 nS/pF, respectively, P < 0.05) (Figure 9A and Supplemental Tables 5 and 6). Half-activation and inactivation voltages
wide association (PheWAS) study on the $\textit{ETV1}$ SNP rs9639168 (missense, S100G) and 1,804 phenotypes in 26,256 individuals of European ancestry (EA) and 3,269 of African ancestry (AA) from the Vanderbilt BioVU biobank, following previously validated methods (46). The phenotype with the strongest association in AA individuals was left bundle branch block (OR = 2.53, $P = 0.0005$; Figure 10A and Supplemental Table 11). Similarly, “other heart block” (ICD9: 426.6) was the strongest phenotype in EA individuals (OR = 2.85, $P = 0.0004$; Figure 10B and Supplemental Table 12).

We then evaluated the effect of this SNP on the subset of individuals who also had available ECGs and found similar results. Associations were seen between rs9639168 and bundle branch block, left bundle branch block, and left anterior fascicular block ($P < 0.05$; Supplemental Tables 13 and 14). The difference in the signals between EA and AA populations suggests that rs9639168 may not be causal but rather be a marker of other variants in $\textit{ETV1}$ that likely have different linkage disequilibrium patterns. Interestingly, “lack of coordination” (OR = 1.64, $P = 0.0005$, AA), also identified in PheWAS analysis, mirrors the neuromuscular phenotype of $\textit{Etv1}$ KO mice, which exhibit severe motor discoordination (38).

Human PheWAS analysis identifies an association between $\textit{ETV1}$ and bundle branch block and heart block. Based on the importance of $\textit{ETV1}$ in establishing and maintaining the fast conduction hierarchy in the heart, we next sought to identify associations between $\textit{ETV1}$ and human conduction disease. We performed a phenome-
Discussion
This study identifies ETV1 as a critical factor essential for transducing endocardially derived NRG1 signals into a transcriptional program responsible for fast conduction gene programming in the heart. ETV1 is a known target of ErbB2 and MAPK signaling pathways and has been implicated in specification, patterning, and functional modulation of various cell types (37, 38, 40). In the gastrointestinal tract, ETV1 is necessary both for proper development of myenteric and intramuscular interstitial cells of Cajal and for malignant transformation of these cell types into gastrointestinal stromal tumors (47). In the central nervous system, ETV1 has been shown to regulate terminal differentiation of cerebellar granule cells (39) and dopaminergic neurons (48). ETV1 has a direct role in establishing the sensory-motor circuitry in the developing spinal cord (38) and also dynamically modulates the electrophysiological properties of postmitotic fast-spiking interneurons through transcriptional regulation of K,1,1 channels (57), the activity of which is also known to be regulated by NRG1/ErbB4 (49). Our data now implicate ETV1 as a major regulator of cardiac conduction biology and place it in the context of NRG1 signaling in the heart. Through NRG1-dependent activation of the Ras-MAPK pathway, ETV1 expression and activity are confined to subendocardial atrial and Purkinje myocytes, where ETV1 orchestrates the expression of the fast conduction gene program.

Etv1 expression is tightly regulated during development in a tissue-specific manner. High levels of ETV1 are detectable in the heart, brain, and lung, while expression levels are low in skeletal muscle and liver (50, 51). In mammalian and amphibian tissues, Etv1 expression is regulated by peptide growth factors (52, 53). In Xenopus animal caps, the peptide growth factors fibroblast growth factor (FGF), bone morphogenetic protein 4 (BMP4), and activin were all able to drive transcription of etv1 (53). During murine cerebral cortex development, Etv1 expression is regulated by FGF8 signaling in Cajal-Retzius cells in the rostral telencephalon (52). In human and murine breast cancer cells, ETV1 expression levels correlate with ErbB2 overexpression and malignant transformation (32, 54). Therefore, the identification of NRG1 as an induction agent for Etv1 in mammalian hearts is in keeping with its known regulation in other tissue types and ensures that Etv1 expression is confined to cardiac regions with high levels of NRG1 exposure, namely the PAM and VCS. Interestingly, ETV1 has been shown in some cellular contexts to induce expression of ErbB2 (55), providing a potential mechanism for reinforcing NRG1-dependent signaling in these myocardial regions.

Activation of NRG1 and MAPK signaling has been shown to positively correlate with fast conduction gene expression in primary and stem cell–derived cardiomyocytes as well as in embryonic heart culture assays (56–59). Conversely, blockade of NRG1 signaling was associated with reduced levels of Scn5a transcript in human embryonic stem cell–derived cardiomyocytes (59). Our work validates these findings and places them in the context of an NRG1-MAPK-RSK/MSK signaling pathway that converges on ETV1 to drive enrichment of Nkx2-5, Gja5, and Snc5a in a tissue-specific manner. NRG1 signaling achieves
highly restricted enrichment of these fast conduction genes in PAM and VCS myocytes through dual regulation of ETV1 expression level and phosphorylation state.

When placed in the context of previously published work from Nkx2-5 mutant mouse models, our data indicate that ETV1 orchestrates fast conduction physiology in atrial and Purkinje myocytes through Nkx2-5–dependent and –independent mechanisms. Etv1 KO hearts displayed an approximately 50% reduction in Nkx2-5 RNA and protein levels specifically in atrial and Purkinje myocytes, which resulted in a corresponding approximately 50% reduction in Cx40 levels and VCS hypoplasia. Cx40 is a known direct transcriptional target of NKX2-5 (8, 60) and responds in a dose-dependent manner (9).

Etv1-null mice displayed structural and functional defects of the VCS that phenocopy Nkx2-5 haploinsufficient mice (18, 44). Meysen et al. attributed the functional defects of the VCS in Nkx2-5 +/– mice to highly restricted enrichment of these fast conduction genes in PAM and VCS myocytes through dual regulation of ETV1 expression level and phosphorylation state.

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Figure 9. Loss of Etv1 homogenizes sodium channel biophysical properties between ventricular, atrial, and Purkinje myocytes. Whole-cell patch clamp was performed on dissociated cardiac cells (ventricular, atrial, Purkinje myocytes) from P18 Etv1 WT and KO mice in a Cntn2EGFP+/– background. (A) Comparison of sodium current–voltage (I–V) relationship. (B) Voltage dependence of steady-state activation. (C) Voltage dependence of steady-state inactivation. (D) Time course of recovery from inactivation. (A–D) Maximum conductance, voltage at half activation (V0.5, activation), voltage at half inactivation (V0.5, inactivation), and tau of recovery (τrecovery) were used to assess significant differences among experimental groups (right panels), respectively (n = 4 hearts). Diagrams outlining patch clamp protocols are included for each endpoint. Data represent mean ± SEM. *P < 0.05, 1-way ANOVA.
the morphological abnormalities of the His-Purkinje system, as mutant Purkinje cells displayed normal action potential properties (44). In agreement with these findings, a perinatal Nkx2-5 KO mouse model displayed normal levels of Scn5a in atrial and Purkinje myocytes (19). In contrast, Etv1 KO mice displayed a reduction of Scn5a RNA and protein levels in atrial and Purkinje myocytes, resulting in homogenization of Na,1.5 throughout the heart. The loss of cell type–specific biophysical properties of the sodium current in Etv1 KO hearts suggests that ET1 regulates Scn5a and other genes known to modulate the sodium current. Thus, the observed conduction slowing in Etv1 KO hearts is likely to reflect alterations in active (ionic) and passive (gap junctional) conductance as well as VCS hypoplasia in the His-Purkinje system.

The identification of associations between an ETV1 sequence variant (rs9639168) and bundle branch block in African Americans and heart block in European Americans points to a potential role of ETV1 in fast conduction gene programming in humans. Whether rs9639168 directly or indirectly affects ETV1 function remains to be determined. The observation that NRG1 signaling blockade downregulates ETV1 expression and results in conduction abnormalities in postnatal hearts points to a continued dependency of NRG1 signaling to maintain the ETV1 fast conduction gene program. Whether there is an age-dependent degradation of the NRG1-ETV1 signaling axis that ultimately plays a role in senile conduction disease will be particularly interesting.

In summary, using multiple orthogonal approaches, including signal transduction screens, transcriptional profiling, mouse genetic models, cellular electrophysiology, and human genetic analysis, we discovered an NRG1 signaling axis that drives cell type–specific expression and activation of ETV1, conferring a fast conduction phenotype in atrial and Purkinje myocytes. Our studies identify ETV1 as a critical regulator of the fast conduction phenotype and demonstrate the biological importance of this gene in cardiac conduction disease.

**Methods**

*Mutant mice.* CCS-lacZ (21), Cntn2-EGFP BAC transgenic (30), and Etv1-nlz (38) (provided by Thomas Jessell, Columbia University, New York, New York, USA) mutant mice have all been previously described. CCS-lacZ and Cntn2-EGFP mice were maintained in a CD1 genetic background. Etv1-null mice were maintained in a C57BL/6 background. For Purkinje cell morphology imaging and quantification, the Etv1null mouse line was bred into the C57BL/6 background. For functional studies, Etv1null Cntn2GFP mouse lines were backcrossed more than 5 generations into the C57BL/6 background.

*Antibody reagents.* Immunofluorescent antibodies were [target, dilution (species, company, product number)]: Nkx2-5, 1:100 (rabbit, Abcam, ab91196); ETV1, 1:100 (rabbit, Abcam, ab36788); ETV1, 1:200 (goat, Santa Cruz Biotechnology, sc-1953); IRX3, 1:100 (rabbit, Abcam, ab25703); Na,1.5, 1:50 (rabbit, Alomone Labs, ASC-005); Cx40, 1:250 (rabbit, Alpha Diagnostic, Cx40A); CNTN2, 1:40 (goat, R&D Systems, AF4439); //a-actinin (sarcomeric), 1:100 (mouse, Sigma-Aldrich, A7811); phospho-ErbB2, 1:100 (rabbit, Abcam, ab108371); phospho-ErbB4, 1:100 (rabbit, Abcam, ab109273); phospho-p44/42 MAPK (pErk1/2), 1:100 (rabbit, Cell Signaling, 4370S); and mouse anti–phospho–histone H3 (PHH3), 1:100 (mouse, Abcam, 14955). Secondary antibodies were donkey anti-rabbit, 1:500 (Santa Cruz Biotechnology, sc-2784); donkey anti-goat, 1:500 (Santa Cruz Biotechnology, sc-2024); and donkey anti-mouse, 1:500 (Santa Cruz Biotechnology, sc-2099).

Western blot primary antibodies were [target, dilution (species, company, product number)]: Nkx2-5, 1:1000 (mouse, Abcam, ab91196); ETV1, 1:200 (goat, Santa Cruz Biotechnology, sc-1953); anti-phosphoserine, 1:500 (rabbit, Millipore, AB1603); Na,1.5, 1:500 (rabbit, Alomone Labs, ASC-005); Cx40, 1:1000 (rabbit, Alpha Diagnostic, Cx40A); and vinculin, 1:5000 (mouse, Abcam, ab11941). Secondary antibodies were goat anti-rabbit, 1:1500 (LI-COR, 926-32211); goat anti-mouse, 1:1500 (LI-COR, 926-32220); and donkey anti-goat, 1:1500 (LI-COR, 926-32214).

*Embryonic heart culture assays and inhibitor assay.* E9.5 CCS-lacZ hearts were harvested and cultured in DMEM containing 1% FBS,
penicillin, and streptomycin (GIBCO/Invitrogen) in 24-well culture plates. The recombinant peptide containing the β variant of the epidermal growth factor-like domain of NRG1 (R&D Systems) was added to each well at a final concentration of 2.5 × 10⁻⁶ M (20). Lyophilized NRG1 was reconstituted at 100 μg/ml in sterile PBS. The control group received vehicle (sterile PBS) only. Medium in both conditions was replaced every 12 hours. Cultures were maintained for up to 48 hours. Nonbeating cultures were excluded from analysis.

For kinase inhibitor studies, the following conditions were used: (a) vehicle control, (b) NRG1 alone, (c) kinase inhibitor alone, or (d) NRG1 plus kinase inhibitor. Doses used for kinase inhibitor studies were: PP2 (10 μM) (61), LY294002 (25 μM) (62, 63), PD98059 (50 μM) (64), FR180204 (20 μM) (65, 66), SL0101 (50 μM) (67), H89 (10 μM) (68).

For RNA analysis, E9.5 heart culture assays described above were used to perform qPCR. Four embryonic hearts were pooled within each replicate to increase total RNA recovered and to minimize assay variability.

Whole-mount staining for β-galactosidase activity. Tissues were collected in ice-cold PBS and fixed for 15 minutes (embryonic) or 1 hour (postnatal) in fix solution (2% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40, 0.01% sodium deoxycholate in PBS). The control group received vehicle (sterile PBS) only. Medium in both conditions was replaced every 12 hours. Cultures were maintained for up to 48 hours. Nonbeating cultures were excluded from analysis.

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Western blot analysis. For atrial samples, left and right atrial samples were collected from P18 mice and immediately cryopreserved in liquid nitrogen. Atrial biopsies were homogenized in RIPA buffer containing protease and phosphatase inhibitors (150 mM NaCl, 1% NP-40 or 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl, pH 8.0, and protease and phosphatase inhibitors). Membranes were then incubated with specific primary antibodies diluted in 5% nonfat dry milk. Membranes were then incubated with specific primary antibodies diluted in 5% nonfat dry milk overnight at 4°C. Immunoblots were scanned and quantified using Odyssey Imaging System (LI-COR).

Cultured rat atrial myocytes. Enriched P1 rat atrial myocyte cultures were obtained by enzymatic digestion (trypsin 2.5%/collagenase 1%) as previously described (71). After overnight plating in DMEM plus 10% FBS plus supplements (3 mM pyruvic acid, 2 g/l BSA, 0.5 mg/ml primocin, 15 mM HEPES, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite, 5 μg/ml linoleic acid, 10 μM ascorbic acid), cells were maintained in serum-free DMEM with supplements. The final myocyte cultures contained more than 90% atrial myocytes at a density of 2 × 10⁴ cells per 60-mm dish. NRGI experiments were performed 24 hours after plating. Cells were used for either ETV1 immunoprecipitation assays or immunofluorescence staining after 0, 1, or 3 hours of vehicle or NRGI treatment. Quantification of ETV1 nuclear accumulation in dissociated neonatal rat myocytes was performed per cell using ImageJ software. DAPI channel was used to define total nuclear area.

ETV1 immunoprecipitation assay. Atrial myocyte cultures were washed twice with ice-cold PBS and resuspended in 1 ml lysis buffer (10 mM Tris, pH 8, 1 mM EDTA, 0.5 mM EGTA, 0.5% N-lauryl sarcosine, and protease and phosphatase inhibitors) per 60-mm dishes. Each 1-mL cell resuspension was individually homogenized on ice using Dounce homogenizers (types A and B). Dynabeads M-280 sheep anti-rabbit IgG (Life Technologies, 11203D) was used with ETV1 1:100 (rabbit, Abcam, ab36788) according to the manufacturer-recommended protocol. Western blot analysis was performed using anti-phosphoserine (rabbit) and anti-ETV1 (goat) antibodies. Protein levels were quantified by densitometry (normalized to ETV1) and plotted relative to WT at baseline.

Electrocardiograms. Surface ECGs were obtained using subcutaneous electrodes attached at the 4 limbs, as previously described (72). P18 mice were anesthetized with inhaled 2% isoflurane. Heart rate was monitored, and core body temperature was maintained at 37.5°C using a heated platform and hair dryer throughout the procedure. Mice with heart rates below 400 bpm were excluded from the analysis. Data analysis was performed on VisualSonics Vevo 2100 V1.5.0 software. The following parameters were measured using short axis M-mode: diastolic and systolic left ventricular internal diameter, anterior wall thickness, and posterior wall thickness. From these measurements, left ventricular ejection fraction and percent fractional shortening were calculated within the Vevo software.

VCS whole-mount quantification. Purkinje cell imaging and quantification of EGFP fluorescence were conducted using Cntn2EGFP/+ reporter mice. Ertvlnc/-Cntn2EGFP/+ mice were generated to study the spatial and temporal expression of Ertvl within the cardiac conduction system as reported by EGFP. Hearts were excised, immediately placed in ice-cold PBS, and fixed for 1 hour in the β-galactosidase fixative (see above). For imaging of the left VCS, the left ventricular wall was cut open at the center of the free wall. Free wall edges were pinned down using 30-gauge needles to expose the left ventricular septum. For imaging of the right VCS, the anterior portion of the right ventricular free wall adjacent to the septum was cut to expose the right ventricular septum and free wall Purkinje fiber network. Bright-field and fluorescent images of the hearts were taken using the Zeiss M2Bio microscope equipped with a Zeiss AxioCam Color camera interfaced with Zeiss AxioVision 2012 software. To acquire EGFP- and X-gal-stained composite images, EGFP images were first acquired, and then pinned hearts underwent X-gal staining and were imaged again. Overlay of bright-field and fluorescent channels was done using Photoshop CS6. Littermates were imaged on the same day at comparable magnification, exposure, and light intensity. Area of GFP+ Purkinje cells was measured using ImageJ software in regions on the left ventricular septum, right ventricular septum, and right ventricular free wall. Quantification was normalized to total area as specified.

ErbB2 inhibitor studies. P1 Ertvlnc/- mice were treated with either vehicle (33% DMSO in sterile saline) or AG825 (1 mg/kg) i.p. daily for 7 days. P1 pups were randomly assigned to either the treatment or vehicle group. ECGs were obtained and hearts were X-gal–stained using methods described above.
Phenome-wide association study. To investigate possible human associations with ETV1 variation, we used a population of 26,256 adults (age >18 years) of European ancestry (EA) and 3,269 of African ancestry (AA) who had genotyping on Illumina HumanExome BeadChip version 1.1 and available electronic health record (EHR) data from the Vanderbilt BioVU DNA biobank (73). This platform contained 1 SNP, rs9639168, in ETV1, which corresponds to a serine to glycine in multiple splice variants. The minor C allele of rs9639168 was present in 34% of EA and 19% of AA individuals. We then evaluated all phenotypes defined using a phenome-wide association study (PheWAS) of this SNP in EA and AA individuals using previously described methods (46). Briefly, the method defines cases for more than 1,600 phenotypes by the presence of specific International Classification of Diseases, 9th edition (ICD9) codes on at least 2 different days. Controls for each phenotype are defined as individuals who lack case ICD9 codes and other codes that are related. For example, cases of the “bundle branch block” phenotype are defined with 426.5, 426.50, 426.53, and 426.54, while its controls are defined as absence of the 426–427.99 range of ICD9 codes. We used version 1.2 of the PheWAS code terminology system and the R PheWAS (74) package to calculate the PheWAS and graph results, both of which can be downloaded from http://phecatalog.org. We used logistic regression for each phenotype with 20 cases or more, adjusted for age and sex, assuming an additive genetic model.

Analyzing human ECG phenotypes. After seeing that the PheWAS identified cardiac conduction phenotypes, we then followed up by analyzing these phenotypes as documented on ECGs available in the EHR. We extracted keywords for left and right bundle branch block, left anterior and posterior fascicular blocks, and all bundle branch blocks from the narrative text ECG impressions, which are generated via interaction of the ECG system and cardiologists. Fifty reports for each type of block were reviewed without identifying any false positives. Cases of each phenotype are defined by the corresponding keywords’ having ever been present in their ECG report. We identify 1 control group for all phenotypes, defined as individuals with at least 1 ECG report in their EHR and absence of any aforementioned keywords of any phenotypes. We used logistic regression to study the association of the C allele of rs9639168 with each ECG phenotype, adjusted for age at the last ECG, sex, and number of ECG reports in the EHR. Specific regular expressions used to define each bundle branch block are defined in Supplemental Table 13.

Statistics. Endpoints were compared using 1-way ANOVA or 2-tailed Student’s t test where appropriate. P less than 0.05 was considered statistically significant. Sample size calculations were done using preliminary data to design the experiment for measuring continuous variables. Groups were constructed to detect a 30% difference between experimental and control groups with a power of 90% and a significance level of 0.05. Mean and SEM were reported for each group. Experimental groups were blinded until the endpoints were analyzed. Animal studies were done prior to genotyping, ensuring blinded observations. All animal ECGs and ultrasound data analysis were conducted by 2 operators.

Study approval. All protocols conformed to the Association for the Assessment and Accreditation of Laboratory Animal Care and the NYU School of Medicine Animal Care and Use Committee. Protocols for studies of cadaveric fetal human tissues were approved by the NYU Institutional Review Board.

Author contributions
AS, GIF, and DSP conceived the project, designed the experiments, analyzed the data, and wrote the manuscript. XL carried out cell electrophysiological experiments, under the advice and supervision of MD. HM, LB, JCD, NJC, and DMR carried out PheWAS experiments. AS, FYL, JZ, and DSP performed all other experimental work.

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