Cardiovascular Research Advance Access published July 1, 2013

Cardiovascular Research
doi:10.1093/cvr/cvt133

Junctophilin-2 is necessary for T-tubule maturation during mouse heart development

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Received 28 January 2013; revised 15 May 2013; accepted 21 May 2013

Time for primary review: 17 days

Aims Transverse tubules (TTs) provide the basic subcellular structures that facilitate excitation–contraction (EC) coupling, the essential process that underlies normal cardiac contractility. Previous studies have shown that TTs develop within the first few weeks of life in mammals but the molecular determinants of this development have remained elusive. This study aims to elucidate the role of junctophilin-2 (JPH2), a junctional membrane complex protein, in the maturation of TTs in cardiomyocytes.

Methods and results Using a novel cardiac-specific short-hairpin-RNA-mediated JPH2 knockdown mouse model (*Mus musculus*; αMHC-shJPH2), we assessed the effects of the loss of JPH2 on the maturation of the ventricular TT structure. Between embryonic day (E) 10.5 and postnatal day (P) 10, JPH2 mRNA and protein levels were reduced by >70% in αMHC-shJPH2 mice. At P8 and P10, knockdown of JPH2 significantly inhibited the maturation of TTs, while expression levels of other genes implicated in TT development remained mostly unchanged. At the same time, intracellular Ca^{2+} handling was disrupted in ventricular myocytes from αMHC- shJPH2 mice, which developed heart failure by P10 marked by reduced ejection fraction, ventricular dilation, and premature death. In contrast, JPH2 transgenic mice exhibited accelerated TT maturation by P8.

Conclusion Our findings suggest that JPH2 is necessary for TT maturation during postnatal cardiac development in mice. In particular, JPH2 may be critical in anchoring the invaginating sarcolemma to the sarcoplasmic reticulum, thereby enabling the maturation of the TT network.

Keywords Transverse tubules • E-C coupling • Junctophilin-2 • Developmental biology

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1. Introduction

Transverse tubules (TTs) are invaginations of the sarcolemma occurring at the Z-lines in cardiomyocytes, forming dyadic junctions with the sarcoplasmic reticulum (SR).[1] These dyads, also known as Ca^{2+} release units (CRUs), contain the Ca^{2+} channels involved in excitation–contraction (EC) coupling, a critical process underlying normal cardiac physiology. While TTs are necessary for normal EC coupling and are disrupted in disease,[2–4] little is known about the molecular determinants of TT maturation.[5,6]

Previously, several proteins have been implicated to play a role in the development of the TT system. For example, caveolin 3 (Cav3) has been shown to transiently associate with developing TTs, and knockout of this protein in mice causes disruption of the TT structure in skeletal muscle.[7] Dysferlin (Dysf), a protein known to interact with Cav3 and the dihydropyridine receptor (DHPR), has been shown to localize to developing TTs in regenerating skeletal muscle.[8,9] Mitsugumin29 (Mg29) has also been implicated in the development of triads within skeletal muscle,[10] but considerably less is known about the proteins required for TT maturation in cardiac myocytes.

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Recently, 'bridging integrator-1' (Bin1) has been shown to be responsible for membrane invagination as part of TT biogenesis in muscle cells and for trafficking of critical Ca^{2+} handling proteins to the CRUs such as the voltage-gated L-type Ca^{2+} channel (LTCC) in the heart.[11,12] Furthermore, Bin1 expression in non-muscle cells was sufficient to cause plasma membrane invaginations that resemble TTs.[12] Although the loss of Bin1 causes hypertrophic cardiomyopathy and perinatal lethality in Bin1-deficient mice,[13] it remains unclear whether Bin1 is required for TT maturation in cardiomyocytes. Finally, junctophilin-2 (JPH2) is a structural protein that localizes to cardiac CRUs and approximates the sarcolemma and the SR membrane. Our recent studies revealed that JPH2 is necessary for maintenance of CRU integrity, and that JPH2 facilitates the communication between LTCCs and ryanodine receptors type 2 (RyR2) on the SR.[4]

Here we describe an important and novel role for JPH2 in the maturation of the TT network using a new mouse model that expresses a cardiac-specific shRNA targeting JPH2 to knockdown JPH2 levels during embryonic development (αMHC-shJPH2). In wild-type (WT) mice, TT development begins by P10, however, perinatal JPH2 knockdown in the αMHC-shJPH2 mice prevented TT maturation. This was also associated with slow, attenuated Ca^{2+} transients caused by delayed and asynchronous subcellular activation of CRUs. In the absence of JPH2, αMHC-shJPH2 mice developed congestive heart failure by P10 characterized by reduced ejection fraction (EF), ventricular dilation, and premature death. Although Bin1 was found to be downregulated at P10, a time-point accompanied by severe heart failure, Cav3, Dysf, and Mg29 were found to be unaltered in this model, suggesting that JPH2 was responsible for these findings. Additional studies in mice with cardiac-restricted JPH2 overexpression revealed accelerated TT network maturation, suggesting that postnatal TT maturation critically depends on JPH2 levels. Together, our data suggest that the structural dyadic protein JPH2 is necessary for the maturation of TTs during early postnatal cardiac development.

2. Methods

Further details are provided in the Supplementary material online.

2.1 Study animals

All protocols involving animals were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. Adult mice and pregnant females were anaesthetized using 2% isoflurane in 95% O_2 prior to cervical dislocation. Embryos and pups up to P10 were decapitated prior to study. All animals used for experiments were *Mus musculus* maintained on a C57BL/6 background for more than 10 generations. Both sexes were used approximately equally for the experiments. To achieve cardiac-specific knockdown of JPH2, a previously generated transgenic mouse expressing shRNA targeting JPH2 (shJPH2)[4] was crossed to transgenic mice expressing Cre recombinase driven by the αMHC promoter (αMHC-Cre).[14] Double transgenic mice (αMHC-shJPH2) and their single transgenic (αMHC or shJPH2) and non-transgenic littermates were used for experiments.

2.2 Real-time PCR

Total RNA was isolated using Direct-zol™ RNA MiniPrep (Zymo Research, Irvine, CA, USA) and quantified using NanoDrop spectrophotometry (Wilmington, DE, USA). Reverse transcription and real-time PCR are described in the Supplementary material online.

2.3 Western blotting

Protein extraction and western blotting were performed as previously described.[4] Details are provided in the Supplementary material online.

2.4 Transthoracic echocardiography

Echocardiographic measurements were performed on unanaesthetized restrained pups. Their cardiac function was measured using M-mode echocardiograms acquired with a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada), as described previously.[15] Details are provided in the Supplementary material online.

2.5 Statistical analysis

Data are presented as mean ± SEM. Student's *t*-test was used for comparison between WT and αMHC-shJPH2 measurements. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1 Cardiac-specific knockdown of JPH2 using shRNA

Because germline knockout of JPH2 is embryonic lethal,[16] we chose to knockdown JPH2 in a cardiac-restricted manner. A previously generated mouse line was used that expresses a transgene composed of an shRNA targeting JPH2 (shJPH2) driven by a U6 promoter, inactive due to the presence of a loxP-flanked *neo* cassette (*Figure 1A*).[4] To obtain cardiac-specific expression of the shRNA, the shJPH2 mice were crossed to mice expressing Cre recombinase driven by the αMHC promoter (αMHC-Cre, or αMHC) (*Figure 1A*).[14] The double transgenic offspring (αMHC-shJPH2 mice) are expected to only express JPH2 shRNA in cardiomyocytes.

We first confirmed that the αMHC promoter turned on in the αMHC-shJPH2 mice. Expression of αMHC was detected as early as embryonic day 10.5 (E10.5), consistent with previous reports (Supplementary material online, *Figure S1*).[17] In addition, there was no difference in the expression of αMHC comparing WT and αMHC-shJPH2 littermates. Next, expression levels of mature JPH2 siRNA were measured using a novel strategy based on real-time PCR (RT–PCR) principles used for microRNAs (miRNAs) (Supplementary material online, *Figure S2*). Robust and specific expression of the mature JPH2 siRNA was detected in cardiac tissue from αMHC-shJPH2 mice as early as E10.5, but not in the WT control mice (*Figure 1B*). Next, the level of JPH2 mRNA was measured to verify knockdown efficiencies. Using RT–PCR, we found that JPH2 was significantly knocked down by more than 70% at all time-points (*Figure 1C*). As expected, knockdown was specific to JPH2, since mRNA levels of JPH1 in the same tissue samples were not reduced in the αMHC-shJPH2 mice (Supplementary material online, *Figure S3*). Finally, protein levels of JPH2 measured using western blotting were also found to be significantly reduced in the hearts of αMHC-shJPH2 mice (*Figure 1D* and *E*).

3.2 Knockdown of JPH2 prevents TT maturation in cardiomyocytes

Based on previous studies, which established that JPH2 links the sarcolemma with the SR,[4,16] we hypothesized that JPH2 knockdown would prevent TT maturation because the invaginating sarcolemma could not be anchored to the SR during cardiac development.[18] TT staining using di-8-ANEPPS revealed the sprouting of early sarcolemmal invaginations at P5 and branching but not yet fully matured TTs in ventricular

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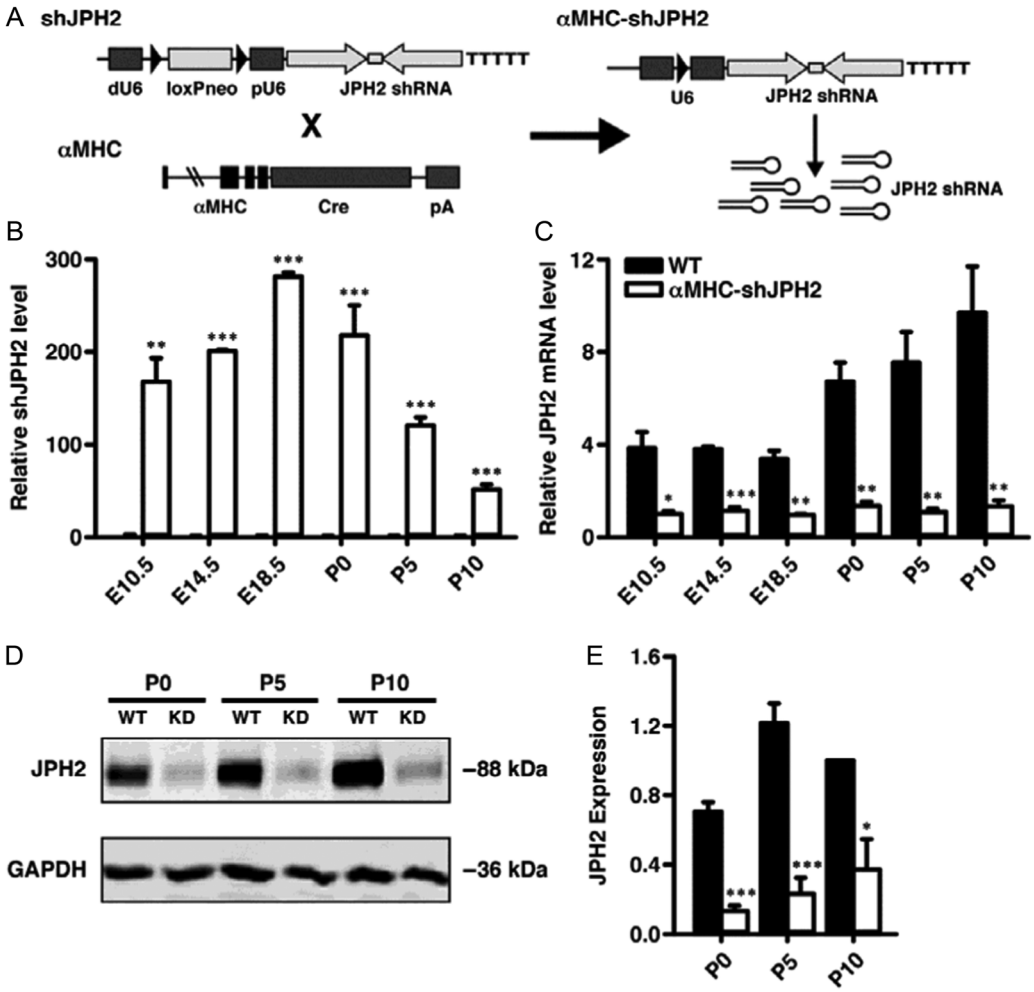

Figure 1 Cardiac-specific knockdown of JPH2 in transgenic mice. (*A*) Mice expressing a transgene composed of an shRNA targeting JPH2 (shJPH2) driven by an interrupted U6 promoter were crossed to mice expressing Cre recombinase driven by the αMHC promoter. The double mutant offspring (αMHC-shJPH2) mice expressed shJPH2 in a cardiac-restricted manner (modified from van Oort *et al.*[4]). Relative level of (*B*) mature siRNA derived from the shJPH2 transgene and (*C*) JPH2 mRNA in WT and αMHC-shJPH2 littermates from embryonic day 10.5 (E10.5) to postnatal day 10 (P10). (*D*) Representative western blots and (*E*) corresponding quantifications showing significant knockdown of JPH2 in αMHC-shJPH2 mice from P0 to P10. $n = 3–5$ mice for all groups; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. WT.

myocytes from WT mice at P10 (*Figure 2A* and *B*; Supplementary material online, *Figure S4*). Similar levels of sarcolemmal invaginations were seen in myocytes from αMHC-shJPH2 mice at P5, although branching of the early TTs appeared to be absent at P10 (*Figure 2A* and *B*). Quantification of TT organization using TT power confirmed that while there was no significant change at P5 (WT: 0.016 ± 0.008 vs. αMHC-shJPH2: 0.015 ± 0.008; $P =$ N.S.), the maturation of TTs were significantly stunted in αMHC-shJPH2 mice at P10 compared with WT littermates (WT: 0.078 ± 0.007 vs. αMHC-shJPH2: 0.026 ± 0.004; $P < 0.001$; *Figure 2C*). Since TTs are largely missing or disorganized at these ages, we devised another way to more accurately quantify the density of the TT network by using TT area % (Supplementary material online). Applying this method showed a similar trend as TT power at P5 (WT: $7.67 \pm 1.11\%$ vs. αMHC-shJPH2: $9.72 \pm 1.23\%$; $P =$ N.S.) and P10 (WT: $21.43 \pm 1.30\%$ vs. αMHC-shJPH2: $14.19 \pm 1.65\%$; $P < 0.001$; *Figure 2D*). Furthermore, by plotting TT power against TT area % for cells from P10 animals, we discovered a previously unidentified population of myocytes from αMHC-shJPH2 mice where the TTs are present but disorganized (*Figure 2E* and *F*). These cells exhibited a high TT area % but a low TT power, consistent with the ability of TTs to extend in the absence of JPH2, but inability to anchor and organize. Another population of cells exhibited a low TT area % as well as low TT power, and likely represents cells at a later stage where TTs have already degraded. Together, these findings suggest an important role for JPH2 in the postnatal maturation of the TT network in cardiac myocytes.

3.3 Developmental expression profiles of other genes implicated in TT formation

Several other genes have previously been implicated in TT formation in skeletal or cardiac muscle, including Bin1, Cav3, Mg29, and Dysf.[1,12,19] Therefore, we next determined whether their expression levels correlated with the stunted TT maturation in αMHC-shJPH2 mice. RT–PCR analysis of Bin1 mRNA levels revealed a peak at E14.5 during embryonic development and a secondary increase starting at P5 in WT mouse

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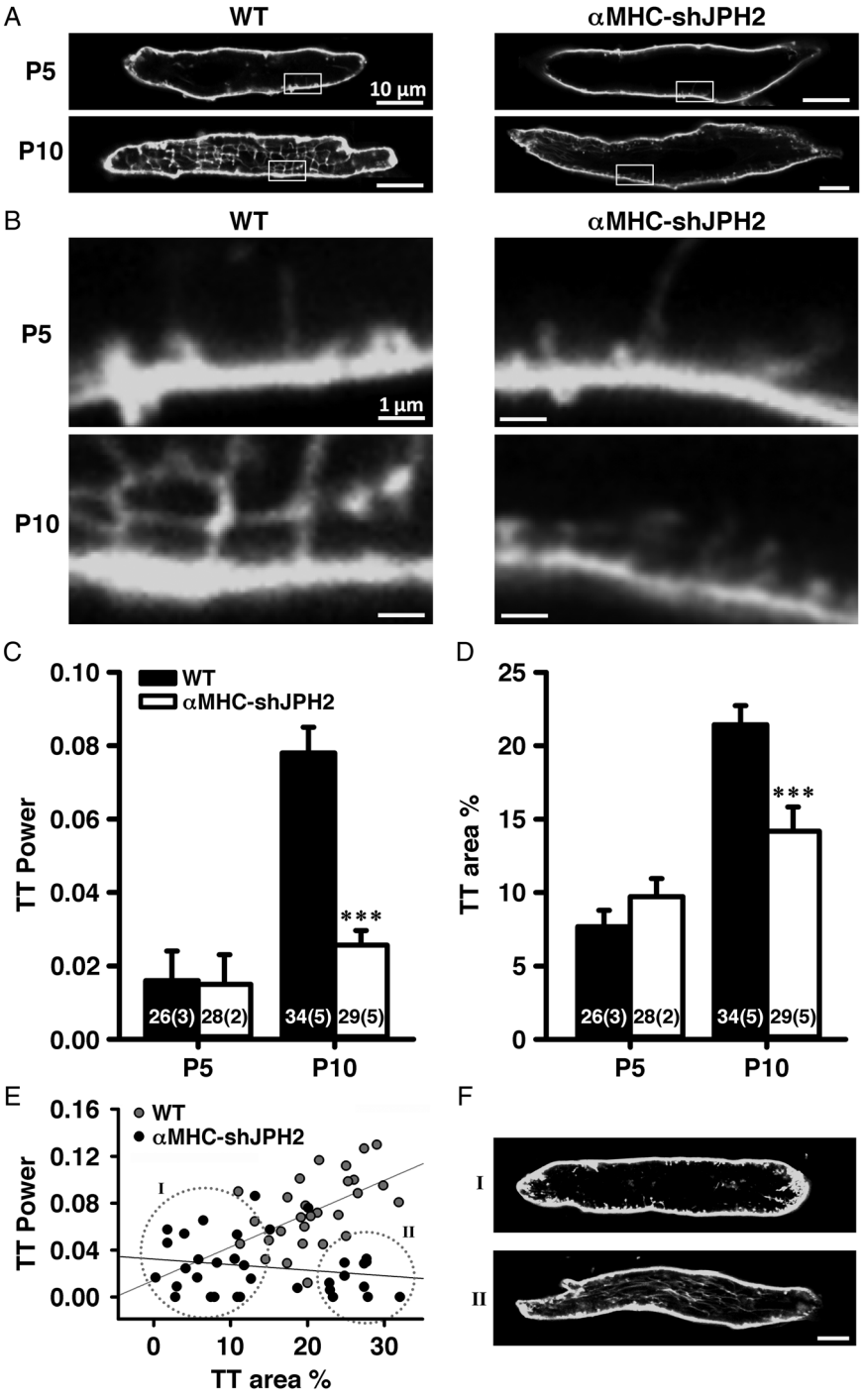

Figure 2 Knockdown of JPH2 prevents formation of TTs in cardiomyocytes. (*A*) Di-8-ANEPPS staining of ventricular myocytes isolated from WT and αMHC-shJPH2 mice at P5 and P10. (*B*) Magnification of red boxes in (*A*) shows the normal formation of TTs in WT myocytes and stunted development of TTs in αMHC-shJPH2 myocytes. Quantification of TTs using both TT power (*C*) and TT area % (*D*). (*E*) Correlation between TT power and TT area % of ventricular myocytes isolated from P10 wild-type (WT) and αMHC-shJPH2 mice and stained with Di-8-ANEPPS. Each circle represents one cell. (*F*) Representative TT staining of cells from two subpopulations of αMHC-shJPH2 cells (I and II). II represents a cell from the subpopulation where TTs are present but disorganized and mostly horizontal. $n = 5$ animals in each group. Scale bar = 10 μm. Numbers in the bars represent the number of cells and numbers in parentheses represent the number of animals. ***$P < 0.001$ vs. WT.

hearts (*Figure 3A*). Bin1 mRNA levels were similar in αMHC-shJPH2 and control littermates throughout cardiac development, with the exception of P10. Since JPH2 knockdown is already prevalent at P5 (*Figure 1C*), the changes in Bin1 mRNA levels at P10 are likely secondary to myocyte remodelling induced by JPH2 knockdown as well as TT degradation. Western blotting also revealed similar Bin1 protein levels in WT and αMHC-shJPH2 mice (*Figure 3B* and *C*). Supporting the notion that JPH2 is an essential component of TT maturation, the mRNA

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levels of other proteins like Cav3, Mg29, and Dysf were all unchanged at P0 and P10 in αMHC-shJPH2 mice (*Figure 3D–F*). Finally, both the protein levels and subcellular localization of key Ca^{2+} handling proteins such as LTCC, NCX1, and RyR2 were not altered in αMHC-shJPH2 mice compared with WT mice (Supplementary material online, *Figures S5* and *S6*). Thus, it is likely that changes in JPH2 alone are sufficient to perturb TT anchoring and maturation in αMHC-shJPH2 mice.

3.4 JPH2 knockdown causes abnormal Ca^{2+} transients

It has been shown that loss of TTs leads to defective intracellular Ca^{2+} release and impaired EC coupling in adult ventricular myocytes.[4] Therefore, we expected that EC coupling would be disrupted in neonatal cardiomyocytes from αMHC-shJPH2 mice, in which the TT network failed to mature. Confocal Ca^{2+} imaging of ventricular myocytes isolated from P10 mice revealed decreased Ca^{2+} transient amplitude in αMHC-shJPH2 mice (0.31 ± 0.03 ΔF/F0) compared with WT mice (0.56 ± 0.05 ΔF/F0; $P < 0.001$; *Figure 4A* and *B*). The time-to-peak (TtP) of these Ca^{2+} transients was increased in myocytes from αMHC-shJPH2 mice (50.14 ± 3.52 ms) compared with WT (26.10 ± 2.41 ms; $P < 0.001$; *Figure 4C*). The SR Ca^{2+} content measured using a caffeine dump protocol (*Figure 4D*) was decreased in myocytes from αMHC-shJPH2 mice (2.78 ± 0.27 F/F0) compared with cells from WT mice (6.11 ± 0.65 F/F0; $P < 0.01$; *Figure 4E*). Furthermore, profiles of Ca^{2+} transients in 1 μm width slices revealed increased spatial dispersion of subcellular Ca^{2+} release within P10 ventricular myocytes from αMHC-shJPH2 mice (Supplementary material online, *Figure S7A*). TtP of these Ca^{2+} transients was quantified for each horizontal 1 μm slice of the cell, plotted in histograms, and fitted with a Gaussian distribution (Supplementary material online, *Figure S7B* and *C*). The distribution suggested that not only the mean TtP was increased, but also the variability of TtP was increased in P10 αMHC-shJPH2 mice compared with WT controls as quantified in Supplementary material online, *Figure S7D*. Together, these findings suggest that JPH2 knockdown led to asynchronous and delayed SR Ca^{2+} release, leading to decreased Ca^{2+} transient amplitude.

3.5 JPH2 knockdown leads to ventricular dilatation and heart failure in αMHC-shJPH2 mice

A number of previous studies have suggested that decoupling between LTCC on the sarcolemma and RyR2 on the SR can lead to heart failure.[4,20,21] Since JPH2 knockdown prevented formation of a mature TT network in αMHC-shJPH2 mice, we hypothesized that αMHC-shJPH2 mice might develop heart failure. Examination of the explanted hearts from P5 and P10 animals showed gross enlargement of the ventricles of αMHC-shJPH2 mice at P10 (*Figure 5A*). This was corroborated by histological sections stained with haematoxylin and eosin (H&E), which showed dilatation of the ventricles and a significant increase in the anteroposterior diameter of hearts from αMHC-shJPH2 mice (3.44 ± 0.12 mm) compared with WT hearts (2.42 ± 0.02 mm; $P < 0.01$) at P10 (*Figure 5B* and *C*). Furthermore, knockdown of JPH2 also caused premature death in the αMHC-shJPH2 mice between days P10 and P15 (*Figure 5D*). Echocardiography of P10 mice revealed that αMHC-shJPH2 mice showed significantly decreased EFs and increased end-diastolic diameters (EDD) compared with WT littermates (*Figure 5E* and *F*). Post-mortem analysis revealed increased heart weight-to-body weight (HW/BW) ratios in the αMHC-shJPH2 mice (*Figure 5G*), consistent with our histological findings. Finally, cell-length measurement showed that ventricular myocytes isolated from the αMHC-shJPH2 mice were also enlarged (*Figure 5H*). Taken together, these data suggest that the αMHC-shJPH2 mice developed dilated cardiomyopathy and severe heart failure at around P10, which leads to their death within the following 5 days.

3.6 Overexpression of JPH2 leads to accelerated TT maturation

To further confirm the role of JPH2 in TT maturation, we used another mouse model where JPH2 is overexpressed in the heart (JPH2-Tg). TT staining of ventricular myocytes isolated from αMHC-shJPH2 (knockdown), WT, and JPH2-Tg (overexpression) mice showed that while there was no difference at P5, TT maturation was accelerated by P8 (*Figure 6A*). Quantification using TT power confirmed this observation in the JPH2-Tg mice where it was 0.015 ± 0.006 at P5 (P = N.S. vs. WT) and 0.112 ± 0.008 at P8 ($P < 0.01$ vs. WT; *Figure 6B*). TT area % showed a similar trend where it was 7.15 ± 0.69% in P5 (P = N.S. vs. WT) and 22.33 ± 1.35% in P8 JPH2-Tg mice ($P < 0.01$ vs. WT; *Figure 6C*). Taken together, this implies a positive dose-dependent relationship between JPH2 expression and TT organization and density, establishing an important role for JPH2 in TT maturation.

4. Discussion

This study demonstrates the essential role of JPH2 in the maturation of the TT network during early postnatal cardiac development in mice. Using a new cardiac-specific JPH2 knockdown mouse model (αMHC-shJPH2 mice), we demonstrated that shRNA-mediated suppression of JPH2 stunted the maturation of TTs between postnatal days 5 and 10 and was associated with asynchronous subcellular Ca^{2+} release. This eventually led to the development of congestive heart failure and premature death between postnatal days 10 and 15. Moreover, we showed using another mouse model (JPH2-Tg) that overexpression of JPH2 in the heart led to an acceleration of TT maturation. Together, these findings support the notion that JPH2 is both necessary and critical for TT maturation and the maintenance of normal cardiac function during development.

4.1 Importance of TTs in cardiomyocytes

Previously, several studies have demonstrated that electrical stimulation of myocytes lacking TTs induces an initial intracellular Ca^{2+} release at the cell periphery, which then propagates into the cell interior by diffusion.[1,22] This immature pattern of Ca^{2+} release involves mainly the LTCC and the Na^{+}/Ca^{2+}-exchanger (NCX1)[23] located at the sarcolemma. For ventricular myocytes at early postnatal ages, this immature EC coupling is sufficient because of the relatively small cell sizes; however, the synchronization of SR Ca^{2+} release becomes more critical as cardiomyocytes increase in size during development. As the TT network develops postnatally, the pattern of EC coupling also matures with RyR2 taking over a more central role, and a more synchronous SR Ca^{2+} release across the myocyte can be observed.[22] In agreement with the above, our data demonstrate that at postnatal day 10, triggered Ca^{2+} release is significantly more synchronous in WT mouse ventricular myocytes (Supplementary material online, *Figure S7*), consistent with the presence of a more mature TT network (*Figure 2*), even though it is still not fully mature (Supplementary material online, *Figure S4*). In contrast, asynchronous SR Ca^{2+} release occurs when JPH2 expression is

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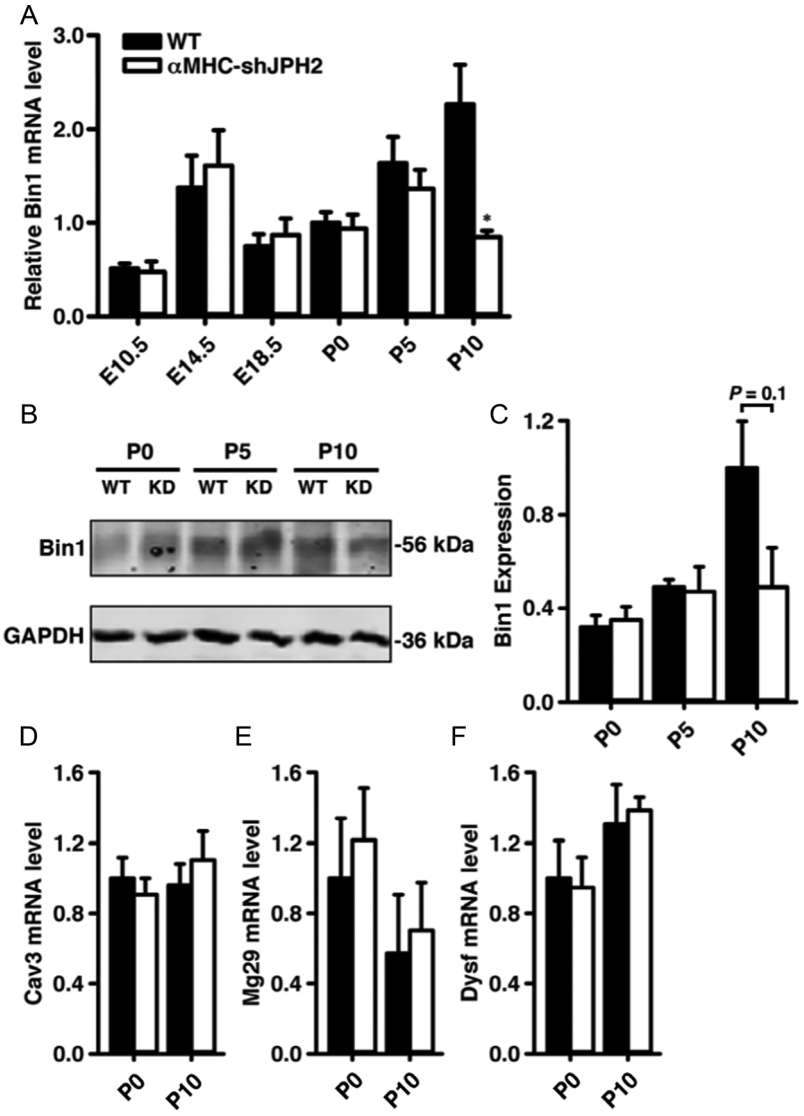

Figure 3 Developmental expression profiles of other genes implicated in TT formation. (*A*) Bin1 mRNA and (*B* and *C*) protein levels at different developmental stages in ventricles of WT and αMHC-shJPH2 mice. (*D*–*F*) Relative mRNA levels of Cav3, Mg29, and Dysf in ventricles of WT and αMHC-shJPH2 mice at P0 and P10. $n = 3$–6 per group. *$P < 0.05$ vs. WT.

suppressed in ventricular myocytes obtained from P10 αMHC-shJPH2 mice, suggesting an important role for JPH2 in developing the subcellular structures required for normal, synchronous EC coupling. It should also be noted that, although the synchronization of triggered Ca^{2+} release and TTs maturation in ventricular myocytes obtained from P10 WT controls are much better than that from age-matched αMHC-shJPH2 mice, neither is comparable to the synchronization observed in WT adult mice. Synchronization of triggered Ca^{2+} release and TTs become fully developed at P20 in WT mice (Supplementary material online, *Figure S4*).

4.2 Early TT development in cardiac myocytes depends on Bin1

TT development begins with invaginations of the sarcolemma, which penetrate the cardiomyocyte interior forming TTs that associate with the SR in structures known as dyads.[24,25] In mice, the formation of the TT network and dyads starts after birth and is finalized during the first 3–4 weeks of postnatal development.[22] Previous studies have demonstrated that initial plasmalemma invaginations are dependent on Bin1 expression,[12] consistent with our finding that αMHC-shJPH2 mice exhibit similar levels of early TT maturation compared with WT at P5 when JPH2 is already significantly knocked down (*Figures 1* and *2*). On the other hand, JPH2 knockdown prevented TT maturation even though Bin1 levels were similar in both WT and αMHC-shJPH2 mice up to P10, at which point the down-regulation of Bin1 may have further contributed to the disappearance of TTs (*Figures 2* and *3*). These findings suggest that while Bin1 is responsible for the initial plasmalemma invaginations, JPH2 is required for the maturation of TTs.

4.3 Maturation of the TT network depends on JPH2

The next step of TT maturation is anchoring of the TT to the SR,[26] which occurs in mice after P5 according to our studies (*Figure 2*). JPH2 has a

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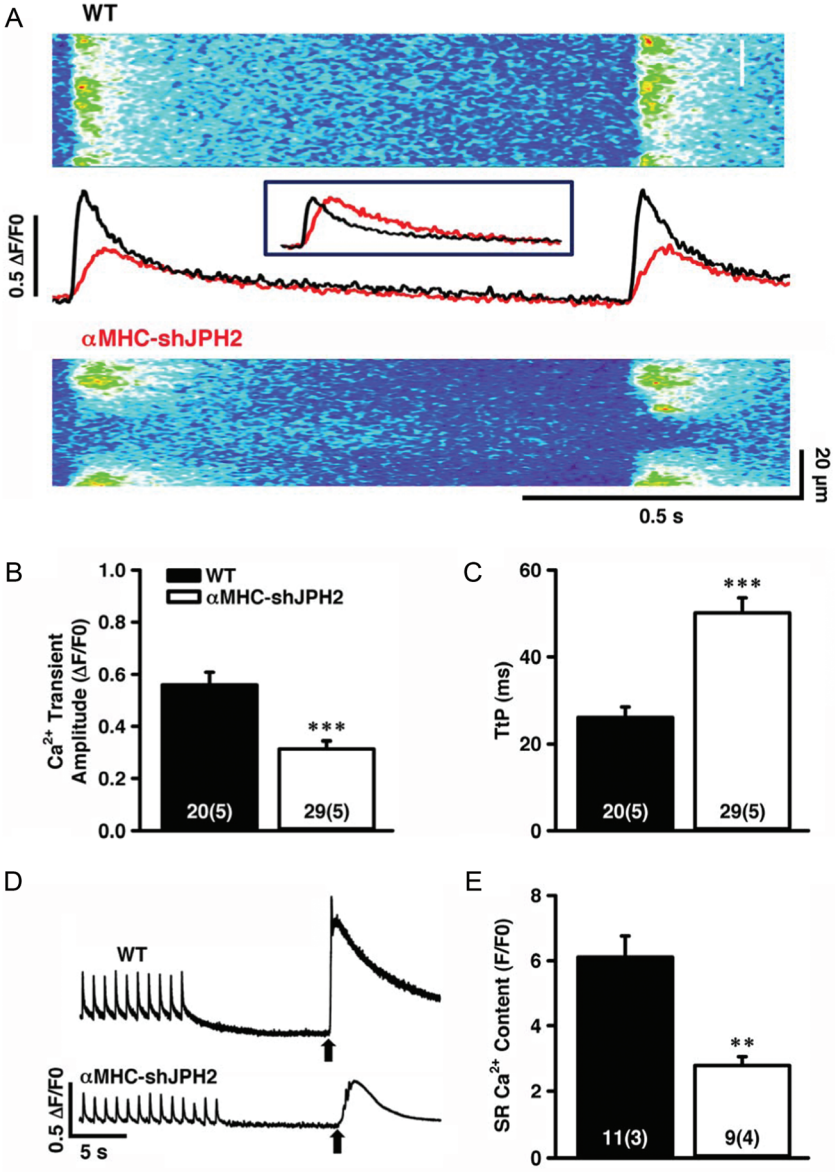

Figure 4 JPH2 knockdown causes abnormal Ca^{2+} transients. (*A*) Representative Ca^{2+} transient recordings demonstrating (*B*) decreased Ca^{2+} transient amplitude and (*C*) increased time-to-peak (TtP) in ventricular myocytes isolated from αMHC-shJPH2 mice at P10. (*D*) Representative tracings of Ca^{2+} transients when cells are paced at 1 Hz and subjected to caffeine (arrows). (*E*) Decreased sarcoplasmic reticulum (SR) Ca^{2+} content in the αMHC-shJPH2 cells. Numbers in the bars represent the number of cells and numbers in parentheses represent the number of animals. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. WT.

transmembrane domain that is embedded in the SR, and it associates with the sarcolemma using MORN domains.[16,27] Based on its sequence,[18] JPH2 is most likely synthesized and trafficked into the ER/SR membrane first, before the MORN domains subsequently recruit other membrane structures, such as the invaginating sarcolemma. Our findings are consistent with a prior study performed in rat ventricular myocytes.[28] Although TT maturation in rats is somewhat delayed compared with mice, a similar pattern of sarcolemma sprouting and anchoring of the TTs to the SR occurs, with a mature TT system being fully established by P20.[28] In the same paper, the authors showed (in *Figure 2* of their paper) that JPH2 appeared in the cell interior earlier than Cav3, which was used as a marker for TTs. This result is similar to our immunocytochemistry data (Supplementary material online, *Figure S6*) and supports the idea that JPH2 arrives at the SR cisternae prior to the formation of dyads, and serves as a target for the growing TTs to anchor. Furthermore, we discovered a population of myocytes in P10 αMHC-shJPH2 mice in which the TTs were present but very disorganized and longitudinal in orientation (*Figure 2E* and *F*). Together, these findings support the notion that while JPH2 may not be involved in sarcolemma invagination, it is necessary for anchoring the early sarcolemmal invaginations to the SR membrane and the formation of dyads, which is a critical step in the maturation of EC coupling.

4.4 Relevance of new shRNA-mediated JPH2 knockdown model

JPH2 was first implicated in the formation and integrity of CRUs in cardiomyocytes based on the finding that germ-line JPH2 knockout mice

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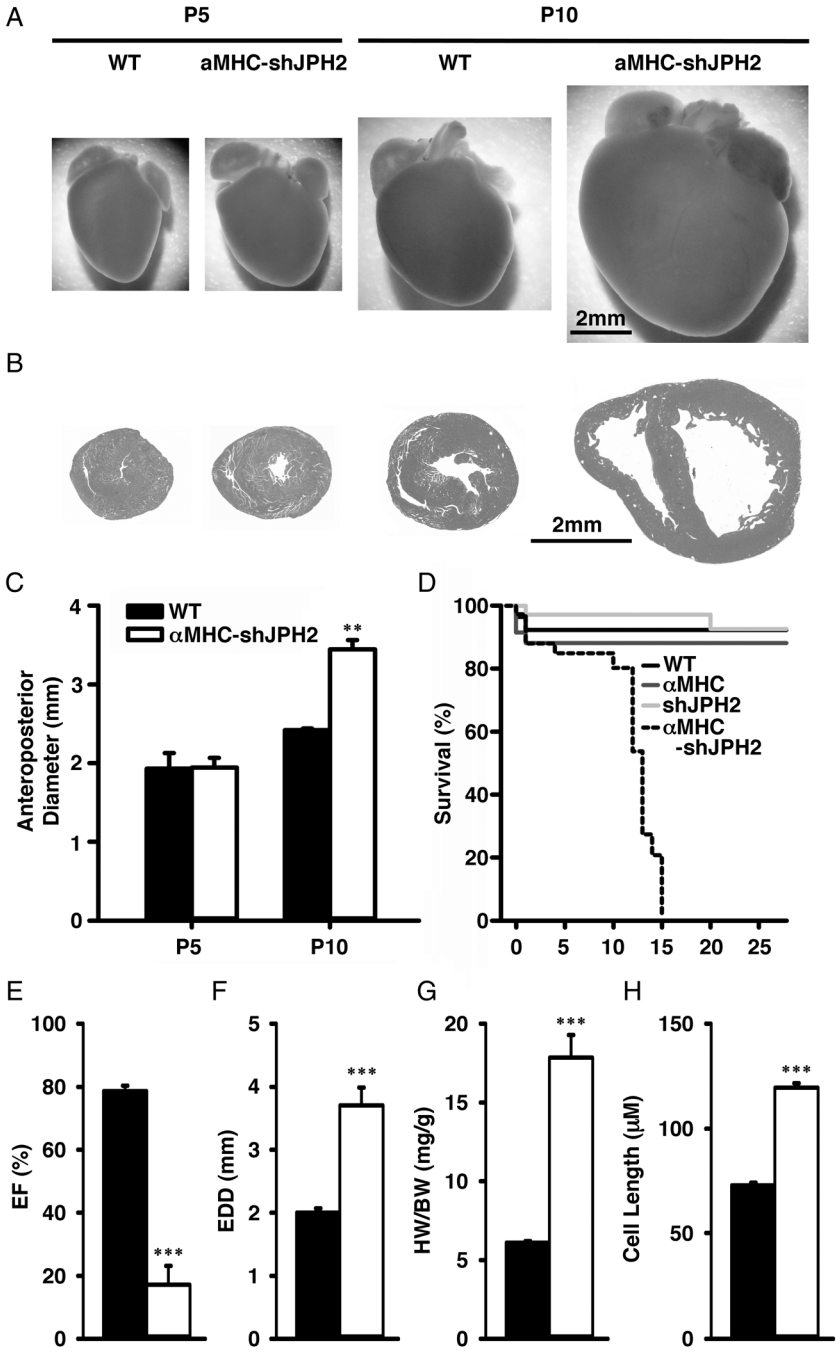

Figure 5 Knockdown of JPH2 leads to ventricular dilation, postnatal heart failure, and mortality in αMHC-shJPH2 mice. (*A*) Representative pictures of explanted hearts from P5 and P10 mice. (*B*) H&E staining of transverse histological sections showing ventricular dilation in αMHC-shJPH2 mouse at P10. (*C*) Quantification of anteroposterior left-ventricular diameters measured from transverse sections, showing a significant increase in P10 in the αMHC-shJPH2 mouse. (*D*) Kaplan–Meier survival curve showing postnatal mortality in αMHC-shJPH2 mice ($n = 37$) compared with WT ($n = 28$) and single transgenic littermates (αMHC, $n = 35$; shJPH2, $n = 40$). (*E*) Echocardiography revealed decreased ejection fraction (EF) at P10 in αMHC-shJPH2 mice and (*F*) and increased end-diastolic diameter (EDD). (*G*) Increased heart weight-to-body weight ratio (HW/BW) in αMHC-shJPH2 mice compared with WT littermates. (*H*) Increased cell length in ventricular myocytes isolated from αMHC-shJPH2 mice compared with WT littermates. (*C*) $n = 3$ in each group; $^{**}P < 0.01$ vs. WT. (*E–G*) $n = 3–11$ mice per group; (*H*) $n = 133$ cells from three WT mice, $n = 106$ cells from five shJPH2 mice; $^{***}P < 0.001$ vs. WT.

failed to form proper JMCs during embryonic development.[27] Electron microscopy analysis of ventricular myocytes from embryonic day E9.5 JPH2−/− embryos revealed fewer JMCs and a greater distance between the plasmalemma and SR membranes.[27] However, it has been impossible to study the role of JPH2 during postnatal development because the germ-line JPH2−/− mice are embryonic lethal by E10.5.[27]

Our new shRNA-mediated JPH2 knockdown mouse model provides non-lethal JPH2 knockdown in the postnatal period during which the TT network matures in mice. Compared with the germ-line JPH2−/−

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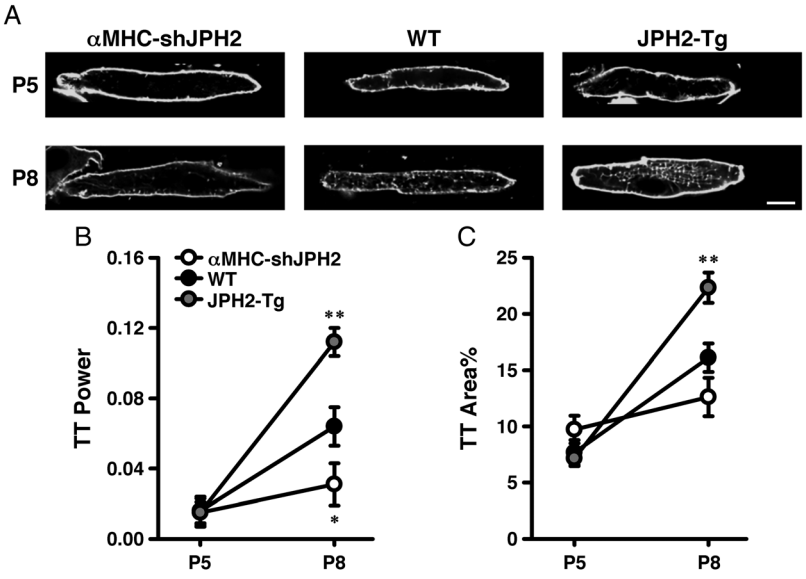

Figure 6 Positive dose-dependent relationship between JPH2 expression and TT development. (*A*) Di-8-ANEPPS staining of ventricular myocytes isolated from αMHC-shJPH2 (knockdown), WT, and JPH2-Tg (overexpression) mice at P5 and P8. TT quantification using both TT power (*B*) and TT area % (*C*) showing an accelerated TT development in JPH2-Tg hearts at P8. Number of cells (and mice) in each group: P5 WT = 26(3), αMHC-shJPH2 = 28(2), JPH2-Tg = 27(2); P8 WT = 19(3), αMHC-shJPH2 = 26(2), JPH2-Tg = 20(3). P5 WT and αMHC-shJPH2 data were re-plotted from *Figure* 2. Scale bar = 10μm. *$P < 0.05$, **$P < 0.01$ vs. WT at the same age.

mice, our model has a cardiac-restricted, incomplete loss of JPH2 and knockdown is temporarily delayed until at least E10.5 when the αMHC promoter becomes active (Supplementary material online, *Figure S1*). However, our model still leads to postnatal lethality by P15 prior to completion of TT maturation. While there is not total loss of JPH2, it has been previously shown that significant reduction in JPH2 within cardiomyocytes elicits disruption of normal cardiac CRUs.[4]

4.5 JPH2 deficiency leads to postnatal HF

Our data show that the failure to form a mature TT network in the absence of JPH2 is associated with the development of dilated cardiomyopathy and heart failure by P10 followed by 100% mortality in αMHC-shJPH2 mice by P15. One likely, though not exclusive, explanation for this finding is that the disorganization and loss of TTs seen in αMHC-shJPH2 mice impairs the maturation of the EC coupling machinery and causes severe contractile failure, which directly leads to heart failure. Similar findings were reported in adult mice in which JPH2 expression was knocked down acutely using the tamoxifen-activated mER-Cre-mER/αMHC-Cre promoter.[4] These adult mice started out with mature TT networks and EC coupling, but the sudden loss of JPH2 induced loss of TTs and impaired Ca^{2+}-induced Ca^{2+} release associated with acute heart failure.[4] Thus, it is likely that JPH2 is essential for the maturation and maintenance of the TT network, as well as robust EC coupling and cardiac contractility.

Alternatively, it is possible that the lack of JPH2 directly affects RyR2 function independently of TT deficits, since JPH2 has been shown to bind to RyR2 and modulate SR Ca^{2+} release in ventricular myocytes.[4] However, this is less likely in neonatal mouse cardiomyocytes since RyR2 does not play the central role until the TTs are more mature.[22] Furthermore, overexpression of JPH2 accelerated TT maturation in ventricular myocytes (*Figure 6*), which also supports the notion that JPH2 plays a key role in TT maturation. Nevertheless, more studies are needed to tease apart these roles of JPH2 during development.

4.6 Limitations

Since complete TT maturation occurs over a period of several weeks in mice (Supplementary material online, *Figure S4*), our mouse model is limited by the postnatal lethality by P15. It is possible that lethality is the direct consequence of the failure to develop a mature TT network in the heart, or indirectly caused by other cellular effects of JPH2 in myocytes. In order to dissect these putative roles of JPH2 in future studies, we propose to use another mouse model, which has an inducible αMHC-driven Cre expression (mER-Cre-mER/αMHC-Cre). This will allow for a delayed onset of JPH2 knockdown and thereby for the investigation of TT maturation without early cardiomyopathy and lethality by P15. Our preliminary studies show that tamoxifen can be administered to pregnant dams or breastfeeding moms to induce JPH2 knockdown at later stages of embryogenesis or early stages of postnatal development, which prolongs their survival. Thus, shRNA-mediated JPH2 knockdown mice present a useful model to manipulate JPH2 expression levels and indirectly the maturation of the TT network during the postnatal period. Another limitation to the current study is that di-8-ANEPPS only stains TTs connected to the sarcolemma and therefore does not allow us to study the small population of TTs that may form internally within the myocyte.[29] Finally, the exact trafficking pathway for JPH2 remains unknown. Based on the most common protein targeting pathways, it is currently assumed that JPH2 is translated within the rough endoplasmic reticulum (ER) and then transported to its anchored position in the SR membrane. Calsequestrin and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), which are two other proteins associated with junctional SR, have been shown to be efficiently trafficked to the SR after synthesis.[30,31]

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4.7 Conclusions

Our findings suggest that JPH2 is necessary for postnatal TT maturation during cardiac development in mice. In particular, JPH2 may be critical in anchoring the invaginating sarcolemma to the SR, thereby enabling the development of mature TTs. The JPH2-dependent formation of a mature TT network is critical for the developing myocyte to transition to a more mature form of EC coupling and normal cardiac contractile function. Overexpression of JPH2 accelerates TT maturation, whereas the loss of JPH2 can lead to disrupted EC coupling and ultimately heart failure in neonatal mice.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: M.J.A. is a consultant for Biotronik, Boston Scientific, Medtronic, St Jude Medical, Inc., and Transgenomic. Intellectual property derived from M.J.A.'s research program resulted in license agreements in 2004 between Mayo Clinic Health Solutions (formerly Mayo Medical Ventures) and PGxHealth (formerly Genaissance Pharmaceuticals, now Transgenomic).

Funding

This work was supported by a W.M. Keck Foundation Distinguished Young Scholar in Medical Research Award, an Established Investigator of the American Heart Association (AHA), and grants from the Muscular Dystrophy Association and NIH (HL089598 and HL091947) (to X.H.T.W.). D.Y.C. and D.L.B. are supported by AHA predoctoral fellowships, and D.L.B. was supported by NIH grant T32HL007676-21A1. M.J.A. is also an Established Investigator of the AHA and is supported by NIH grants HD42569 and HL94291, and the Mayo Clinic Windland Smith Rice Comprehensive Sudden Cardiac Death Program. L.-S.S. is supported by NIH grant HL090905 and AHA scientist development grant 0635056N. This work was also supported in part by Fondation Leducq ('Alliance for CaMKII Signaling in Heart') to X.H.T.W. and M.J.A.

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