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Morphogenesis of T-tubules in heart cells: the role of junctophilin-2

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The T-tubule (TT) system forms the structural basis for excitation-contraction coupling in heart and muscle cells. The morphogenesis of the TT system is a key step in the maturation of heart cells because it does not exist in neonatal cardiomyocytes. In the present study, we quantified the morphological changes in TTs during heart cell maturation and investigated the role of junctophilin-2 (JP2), a protein known to anchor the sarcoplasmic reticulum (SR) to TT, in changes to TT morphological parameters. Analysis of confocal images showed that the transverse elements of TTs increased, while longitudinal elements decreased during the maturation of TTs. Fourier transform analysis showed that the power of ~2 μm spatial components increased with cardiomyocytes maturation. These changes were preceded by increased expression of JP2, and were reversed by JP2 knockdown. These findings indicate that JP2 is required for the morphogenesis of TTs during heart development.

T-tubules, junctophilin-2, cardiomyocytes, morphogenesis

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T-tubules (TTs) of cardiomyocytes are invaginations of the cell membrane. TTs conduct electrical excitation to the close vicinity of myofilaments and play key roles in synchronizing the cell-wide initiation of contraction [1,2]. Most TTs run transversally along Z-lines, with a few longitudinal elements connecting TTs between adjacent Z-lines [3]. The mean diameter of TTs in cardiac myocytes varies from 180 to 280 nm [3,4]. TTs contain a high density of membrane proteins, such as voltage-gated sodium channels, L-type calcium channels and sodium-calcium exchangers, which are required for TT membrane excitation, excitation-contraction (E-C) coupling and intracellular calcium removal [1,5–7]. TTs are widely distributed in cardiac cells and meet the sarcoplasmic reticulum (SR) with ~12 nm junctional clefts, forming structural units for calcium signaling, known as couplons [8,9]. During E-C coupling, the Ca^{2+} influx through L-type calcium channels on the TT activates the ryanodine receptor (RyR) calcium release channels on the adjacent SR to initiate a calcium transient [10,11]. This Ca^{2+}-induced Ca^{2+} release (CICR) process drives the contraction of cardiac cells [12,13].

In mammal cardiomyocytes, peripheral couplons (the junction between the surface of the cell membrane and the SR) already exist during embryogenesis [14,15]. However, the morphogenesis of TTs and the formation of TT-SR couplons begin at a relatively late stage [14,16]. The TT network does not appear until 7–10 days after birth [14,17]. Two possible models for TT development, the invagination model and the pre-association model, have been proposed [14,17]. In the invagination model, TTs originate from the surface membrane and subsequently extend into the center of the cell [14,17]. The SR develops independently and docks with TTs to form junctional dyads. In the pre-association model, TTs initially appear as vesicles coupled

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with SR in the cell, unconnected with the surface membrane. The vesicles then fuse with the plasmalemma to form TTs. Although the exact mechanism of TTs formation is still under debate, the attachment to the SR is no doubt essential to the process.

Junctophilin-2 (JP2) is a structural membrane protein that anchors the SR to the TTs/cell membrane [15]. It is composed of a hydrophobic segment spanning the SR membrane and a cytoplasmic domain interacting with the cell membrane [15,18]. Accumulating evidence suggests that JP2 is critically important in the formation of TT-SR junctions and the healthy operation of CICR between L-type calcium channels and RyRs [19–24]. Absence of JP2 in mice is embryonic lethal [15]. During heart failure, JP2 expression is decreased, leading to reduced density of TT-SR couplons and desynchronized Ca^{2+} release in ventricular myocytes [23,24].

Although it is known that JP2 is important in the formation of TT-SR junctions, whether it is required for the TT morphogenesis is not clear. In the present study, we examined the morphology of TTs and the expression of JP2 in maturing cardiomyocytes, and tested the role of JP2 in TT morphology using RNAi technology.

1 Materials and methods

1.1 Cell preparation and virus infection

Cell isolation was conducted in accordance to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. Rat ventricular cardiomyocytes were enzymatically isolated from the hearts of postnatal Sprague-Dawley rats at different maturation stages (postnatal days 15, 20, 30 and 60), as previously reported [25]. Briefly, rats were anesthetized by intraperitoneal injection with urethane (1000 mg kg^{-1} i.p.). The hearts were rapidly removed and perfused on a Langendorff system. Cardiomyocytes were isolated following collagenase II perfusion. The isolated cells were kept in Tyrode solution containing (in mmol L^{-1}): 137 NaCl, 4.0 KCl, 1.0 $CaCl_2$, 1.2 NaH_2PO_4, 10 glucose, and 10 HEPES, at pH 7.36, adjusted with NaOH.

In the RNAi experiments, isolated cardiomyocytes from day 60 were cultured in M199 medium with 5% CO_2 at 37°C. Adenovirus with shRNA targeted to JP2 or nonspecific sequence was added to the culture medium at an optimized multiplicity of infection (MOI). JP2 siRNA sequence: 5′-ACACCGTCCTCATCTGTAT-3′; control sequence: 5′-TTCTCCGAACGTGTCACGT-3′. Cardiomyocytes of both groups were subjected to imaging and biochemical experiments 48 h after infection.

1.2 Membrane staining and imaging

Isolated cardiomyocytes from rats of different ages were stained with 1 μmol L^{-1} di-8-ANEPPS dye (Invitrogen, USA) in Tyrode solution for 30 min at room temperature. The cells were washed with normal Tyrode solution three times to remove residual dye. In JP2 RNAi experiments, cultured cells were stained with a potentiometric fluorescent indicator, RH237 (Invitrogen, USA). The dye was initially dissolved to 5 mmol L^{-1} in dimethyl sulfoxide (DMSO) and applied to cardiomyocytes at a final concentration of 10 μmol L^{-1} for 5 min at room temperature. TTs images were obtained using an inverted confocal microscope (LSM-710, Carl Zeiss, Oberkochen, Germany) equipped with an argon (488 nm) laser, as well as a 40×, 1.3 N.A. oil immersion objective lens.

1.3 Western blotting

Ventricles from rats at different maturation stages were homogenized in the lysis buffer. Equal amounts of protein (15 μg) in each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The antibody that recognizes JP2 was prepared by AVIVA Bio Co. Ltd (Beijing, China). The membranes were probed with anti-JP2 (1:1000) antibody at 4°C overnight. They were developed using horse radish-peroxidase (HRP)-conjugated antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:20000, KangChen, China) and anti-rabbit secondary antibody (1:1000, Thermo Scientific, USA). Average densities of protein bands were measured using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). JP2 signal densities were normalized to the corresponding GAPDH signal densities.

1.4 Image analysis

TTs images were analyzed using Interactive Data Language 5.5 programming language, as previous reported [23,26]. TTs extending in either the transversal or longitudinal directions were quantitatively analyzed. Fast Fourier transformation (FFT) was used to analyze the regularity of TTs. The power value characterizing the organization of TTs was used to evaluate TT development.

1.5 Statistics

All data are expressed as mean±SEM, as indicated. Statistical analysis was performed using one-way ANOVA or the Student's *t*-test. A P-value <0.05 was considered significant.

2 Results

2.1 TTs formation during rat postnatal development

We used membrane dye di-8-ANEPPS to stain rat cardio-

myocytes at different maturation levels and visualized the cell membrane and TTs by confocal microscopy. According to previous reports, TTs do not appear until 7–10 d postnatally [14,17]. We therefore visualized TT maturation from postnatal day 15 to 60 (Figure 1A). On day 15, TTs were found mainly near the cell surface. Only a few TTs were found in the center of the cells. By day 20, the TT had started to expand to the whole cell, except the nucleus. Transversal striations of TTs across the cell were clearly observed at this stage. Thereafter, a well-organized TT network was widely distributed in cardiomyocytes on postnatal days 30 and 60. During the above maturation process, TTs became orderly aligned, with ~ 2 μm distance between neighboring TTs.

To quantify the regularity of TTs during development, we adopted an FFT algorithm [23,26] to determine the spatial period of TTs (Figure 1B). The dominant spatial period of ~2 μm (arrow in Figure 1B) reflected the physical distance between neighboring TTs in cardiomyocytes. We used the peak power value at the ~2 μm spatial period as a quantitative index of TT organization (TT_{power}) and found that TT_{power} increased gradually during maturation (Figure 1C). The higher TT_{power} in later stages of maturation indicates that TTs were inter-spaced in a more regular manner.

During maturation, while most TTs ran transversally along the Z-lines, a portion of TTs ran longitudinally. To quantify the orientation of TTs during maturation, the density of transverse and longitudinal elements were measured separately (Figure 2A). Punctate elements that could not be judged as transverse or longitudinal elements (e.g., the green square in Figure 2A) were not taken into consideration. We found that the fraction of transverse elements increased (Figure 2B) and that of longitudinal elements decreased (Figure 2C) gradually during the maturation of cardiomyocytes. The changes of both elements approached a steady state after day 30. This finding indicates that not only the inter-TT distance but also the orientation of TTs was unified during maturation.

2.2 The role of JP2 expression in TT morphology

JP2 anchors the SR to TTs/cell membrane in cardiomyocytes [15]. To elucidate the possible role of JP2 in TT morphogenesis, we quantified JP2 expression in rat ventricles at different maturation stages by western blot assay (Figure 3A). The result showed that JP2 expression increased with the maturation of cardiomyocytes and reached a steady level around day 30 (Figure 3B).

To test the relationship between JP2 expression and TT morphology during cardiac maturation, we sought to knockdown JP2 by RNAi technology. We used adenovirus containing the shRNA specific to JP2 to suppress the expression of JP2 in cultured cardiomyocytes at day 60, which contain a mature TT network. The adenovirus vector also contained the sequence of a fluorescent protein, DsRed, allowing us to ensure that >90% of the cardiomyocytes were infected. Two days after the adenoviral infection, Western blot assay showed that the JP2 expression was cur-

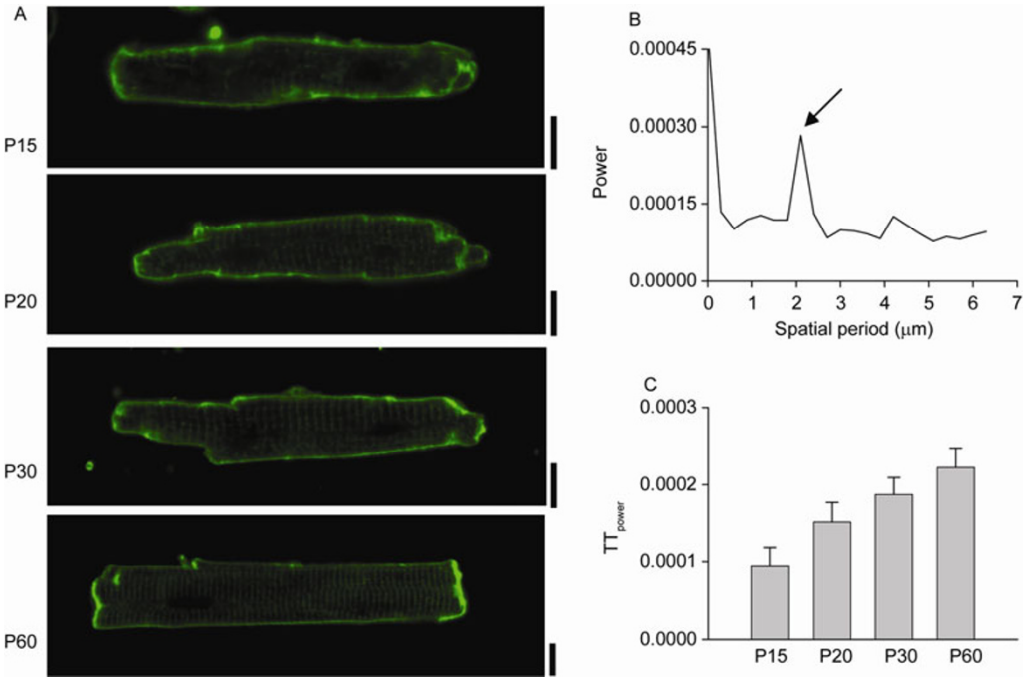

Figure 1 The regularity of TTs during the maturation of cardiomyocytes. A, Confocal images of acutely isolated cardiomyocytes at different maturation stages labeled with the membrane dye, di-8-ANEPPS. Scale bar, 10 μm; P15, postnatal day 15; P20, postnatal day 20; P30, postnatal day 30; P60, postnatal day 60. B, FFT analysis of spatial period. The peak value of power at ~2 μm was defined as TT_{power}, representing the average TT spacing. C, The value of TT_{power} increased with development. (N>15 from three animals for each time point; One-way ANOVA; P<0.01).

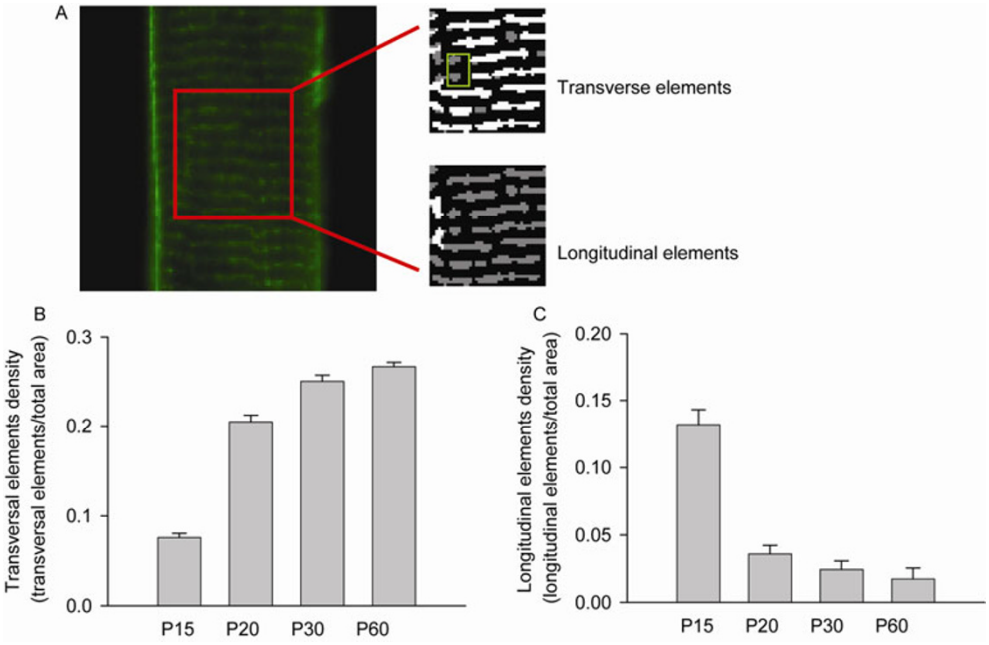

Figure 2 The change in TT orientation during cardiac maturation. A, The TT signals in cardiomyocytes labeled with membrane dye (left) were separated into transverse elements (upper right) and longitudinal elements (lower right). The dots in the green rectangle, whose orientations were not clear, were not taken into consideration. B, The density of transverse elements, calculated by dividing the area of transverse elements by the total area selected, increased with cardiac maturation. C, The density of longitudinal elements decreased with cardiac maturation. (N>15 from three animals for each time point; One-way ANOVA; P<0.01).

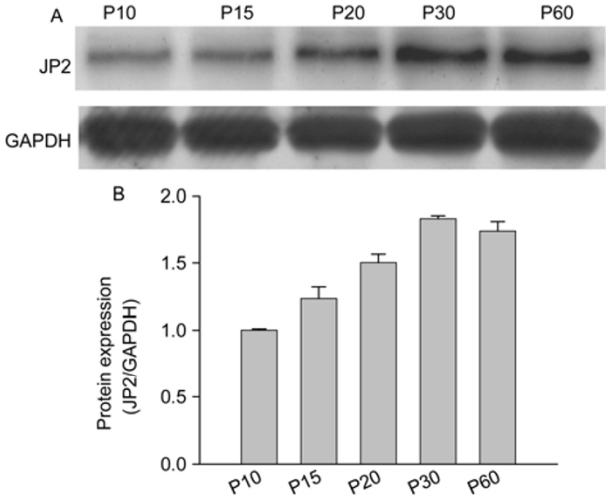

Figure 3 The expression of JP2 during maturation of cardiomyocytes. A, The expression of JP2 protein was analyzed by western blot, with GAPDH as an internal reference. B, The expression level of JP2 increased with cardiac maturation. (N=3 from three animals for each time point; One-way ANOVA; P<0.05).

tailed by ~ 40% in the JP2 RNAi group, compared with the group infected with adenovirus carrying a scrambled shRNA (Figure 4A).

To avoid fluorescence interference by DsRed, we chose to use another fluorescent indicator, RH 237, to stain the cell membrane. We found that JP2 knockdown led to remodeling of the TT structure in day 60 cardiomyocytes in culture (Figure 4B). TTs became disorganized and irregular in the JP2 RNAi group, compared with those in the control group. To quantify the morphological changes, we measured the TT_{power} period using the FFT algorithm. Contrary to what was observed during the maturation process, TT_{power} was significantly lower in the JP2 RNAi group than in the control group (Figure 4C).

We also quantitatively analyzed the fractions of transverse and longitudinal elements in total TTs. JP2 shRNA-infected cardiomyocytes exhibited a significant loss of transverse elements compared with the control group (Figure 5A). In contrast, JP2 knockdown resulted in an increase in longitudinal elements (Figure 5B). All of these changes caused by JP2 RNAi were the reverse of those that occurred during TT maturation, indicating that JP2 is required for the morphological maturation of TTs in cardiomyocytes.

3 Discussion

In the present study, we examined the morphological changes in the TT network and JP2 expression, and tested their relationship during the postnatal maturation of cardiomyocytes. We showed that TT maturation was characterized by increased power of the ~2 μm spatial period by FFT analysis and an increased fraction of transverse elements. These changes were preceded by a corresponding increase in JP2 expression. Because suppression of JP2 expression

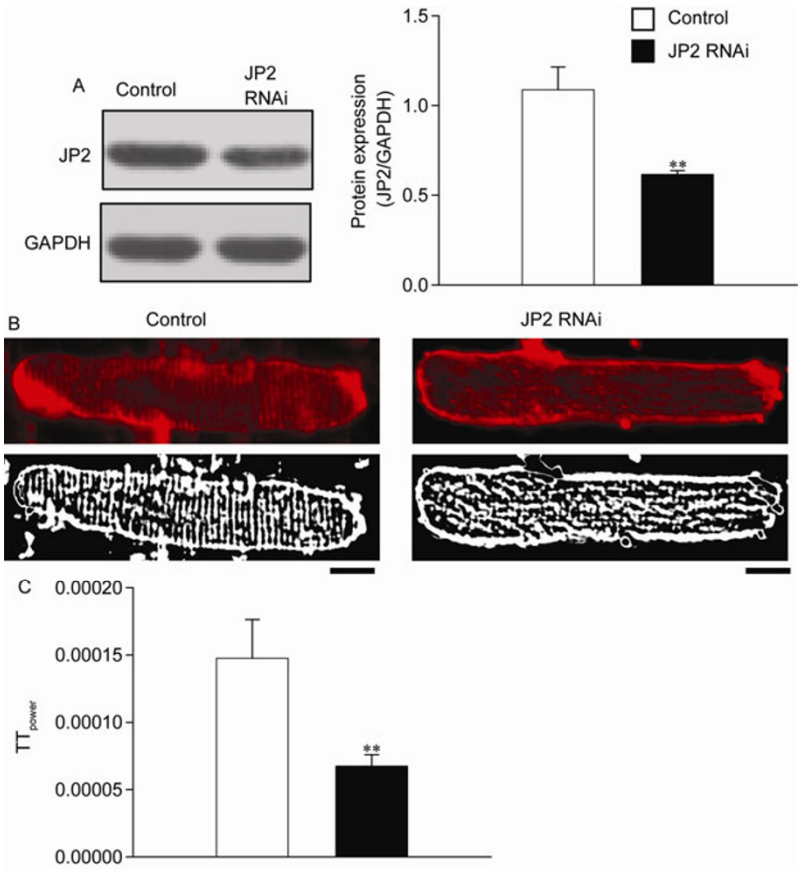

Figure 4 JP2 knockdown decreased the regularity of TTs in cultured cardiomyocytes. A, Western blot assay of JP2 protein in cultured cells infected with adenovirus carrying control (white bar) or JP2 shRNA (black bar). The expression level of JP2 protein in the RNAi group was decreased by about 40% compared with control group (N=3, t-test, **, P<0.01). B, Images of control- (left) and JP2 shRNA-infected (right) cardiomyocytes stained by membrane dye, RH 237. High-contrast images are shown below the original images. Scale bar, 10 μm. C, JP2 knockdown caused reduction of TT_{power} in cultured cardiomyocytes. (N>20, for both the control and JP2 RNAi groups, t-test, **, P<0.01).

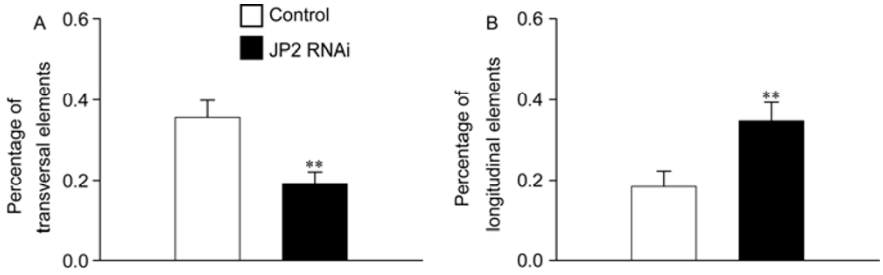

Figure 5 JP2 knockdown altered TT orientation in cultured rat cardiomyocytes. A, JP2 knockdown decreased the fraction of transverse elements. B, The fraction of longitudinal elements was increased by JP2 knockdown. (N>20, for both control and JP2 RNAi groups, t-test, **, P<0.01).

reversed these changes, we concluded that JP2 expression level is an essential factor in the morphological maturation of TTs in cardiomyocytes.

TTs are absent or very rare in embryonic and neonatal cardiomyocytes and are formed during the first few weeks of life [14,17]. The most rapid growth period of the TT network is from day 10 to 20, in which TTs gradually extend from the cell periphery into the cell interior. The present study was focused on the maturation of TTs, and we quantified the TT morphogenesis from day 15 to 60. To our knowledge, this is the first time that the TT morphology during development has been parameterized. This quantification identified two morphological features during maturation: (i) the TT_{power} was increased, reflecting that TTs were gradually docked to the 2 μm-spaced Z-lines; and (ii) the transverse and longitudinal elements were shifted in a push-pull manner, reflecting that TTs extended along Z-lines.

In mammalian ventricular myocytes, the TT network consists of a few longitudinal elements and many transverse

elements [3,4]. This well-organized, regularly distributed TT network is essential for rapid excitation and synchronous Ca^{2+} release throughout the whole cardiomyocyte in response to the action potential [6]. JP2 shRNA-infected cardiomyocytes exhibited decreased regularity of TT spacing, a reduced fraction of transverse elements and an increased fraction of longitudinal elements, indicating that JP2 is involved in the morphogenesis of TTs. Previous reports show that JP2 is involved in the cardiogenesis of embryonic stem cells [27]. Knockdown of JP2 in HL-1 cells results in myocyte hypertrophy [21]. However, it seems that partial silencing of JP2 in cultured adult cardiomyocytes does not alter the morphology of cardiomyocytes [24]. Neither the length nor the width of the cells is changed by JP2 knockdown [24], in close agreement with our results.

In both models of TT development discussed in the introduction, the coupling of TT to the SR is an essential step [14,17]. It is reported that SR terminals are aligned at the Z-line area earlier than TT formation [17,28]. Given that JP2 spans the SR membrane, it is expected that TTs, or cell membrane invaginations, are built on the track of SR terminals, which requires the interaction between JP2 and cell membrane and/or membrane proteins. In this scenario, a sufficient amount of JP2 molecules would be crucial for anchoring TTs to as many SR terminals as possible. In the early stage of development, the JP2 expression level is low. Although peripheral coupling between the SR and cell membrane can be formed [15], there are too few JP2 molecules to anchor TTs throughout the Z-line area. With the development of cardiomyocytes, the more JP2 that is expressed, the more TT-SR junctions are formed; and the more regular the TT network, the more mature the excitation-contraction coupling. Therefore, the level of JP2 expression during the maturation stage of development is critically important to the formation of a well-organized TT network in heart cells.

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