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Cardiac Transverse Tubules in Physiology and Heart Failure

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Abstract

In mammalian cardiac myocytes, the plasma membrane includes the surface sarcolemma but also a network of membrane invaginations called transverse (t-) tubules. These structures carry the action potential deep into the cell interior, allowing efficient triggering of Ca^{2+} release and initiation of contraction. Once thought to serve as rather static enablers of excitation-contraction coupling, recent work has provided a newfound appreciation of the plasticity of the t-tubule network's structure and function. Indeed, t-tubules are now understood to support dynamic regulation of the heartbeat across a range of timescales, during all stages of life, in both health and disease. This review article aims to summarize these concepts, with consideration given to emerging t-tubule regulators and their targeting in future therapies.

1. THE FUNDAMENTALS OF CARDIAC TRANSVERSE TUBULES

1.1. T-Tubule Morphology

Transverse tubule (t-tubule): an invagination of the surface membrane that is rich in ion channels and receptors

Z-line: the boundary between sarcomeres in striated muscles appearing as a dark band in micrographs; a structural anchor point

Sarcoplasmic reticulum (SR): an intracellular interconnected membranous network acting as the principal calcium store in the heart

Junctophilin-2 (JPH2): a dyad-spanning protein tethering RyRs and LTCCs; a calpain cleavage product is a transcriptional regulator

L-type calcium channel (LTCC): a voltage-gated L-type Ca^{2+} channel preferentially located on the t-tubule membrane

Ryanodine receptor (RyR): a tetrameric protein forming organized clusters and functioning as the calcium release channel of the sarcoplasmic reticulum

In the mammalian heart, the surface membrane of cardiac myocytes includes a network of tubules that extend into the cell interior. These transverse (t-) tubules were so named in early electron microscopy studies based on these structures' prominent perpendicular orientation with the long axis of ventricular (and skeletal muscle) cells (1). However, subsequent work has identified that there is in fact significant variability in t-tubule structure within cells, across the heart, and between species. In addition to transverse elements positioned along the Z-lines of each sarcomere, obliquely and longitudinally oriented t-tubules extend into the sarcomere interior (A-band). In the ventricles of small rodents such as mice and rats, the t-tubule network is highly organized, with a dense, interconnected network of transverse and longitudinal components. In larger mammals, t-tubule density is generally lower, with a less-extensive presence of tubules in both orientations. T-tubule dimensions also vary, as electron microscopy studies have reported that t-tubule diameters range between 20 and 450 nm (2, 3). Additional complexity of t-tubule shape is afforded by folding of the t-tubule membrane, which is suggested to limit fluid exchange between the t-tubule lumen and the extracellular space (4). Furthermore, fluid exchange has recently been proposed to be facilitated by deformation of t-tubule morphology during the contraction-relaxation cycle (5).

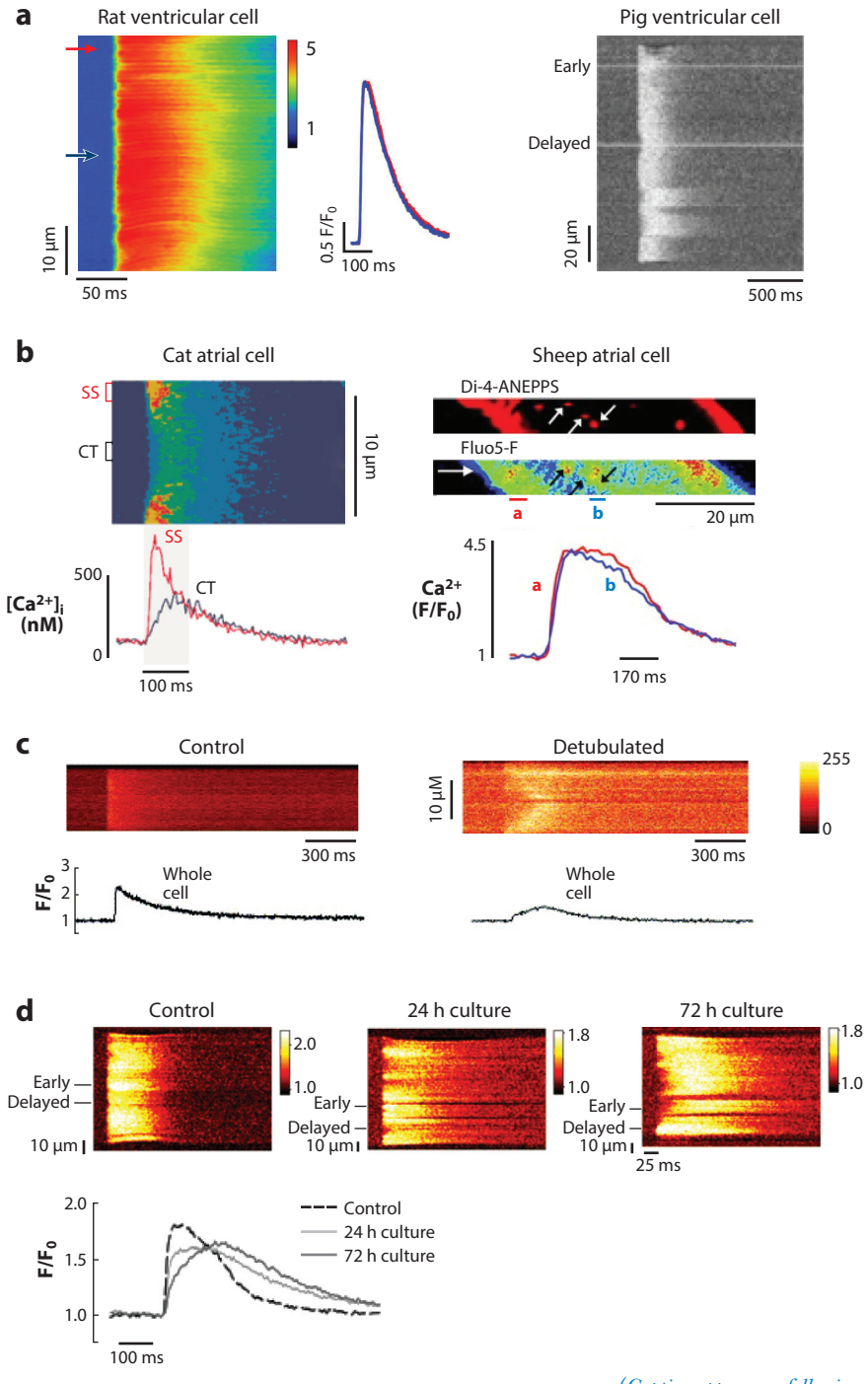
Although it was long thought that atrial myocytes lack tubules, it is now understood that these structures are present in the atria of both small and large mammals (6–9). Nevertheless, t-tubule structure can vary considerably between atrial myocytes; some cells may exhibit rather organized, transversely oriented t-tubules reminiscent of ventricular cells, while in other cells t-tubules may appear in a more disorganized, longitudinal orientation or be absent (8). It is perhaps this variability or technical difficulties associated with the isolation of these cells that contributed to the previous lack of identification of atrial t-tubules in early work.

Accumulating data have indicated that t-tubule structure is highly malleable. This plasticity includes both compensatory and decompensatory alterations that occur throughout life, during health and disease. The molecular drivers and functional consequences of these alterations are discussed in subsequent sections of this review. First, however, we review t-tubule structure and function in the healthy adult heart (10–13).

1.2. T-Tubule Function

Contraction and relaxation of cardiac myocytes, and thus the whole heart, are critically regulated by t-tubules and their functional couplings with the junctional sarcoplasmic reticulum (jSR). Termed dyads, these junctions (also called junctional membrane complexes) are anchored by junctophilin-2 (JPH2), which strictly maintains a cleft width of 12–15 nm (13). Dyads serve as key sites for Ca^{2+} transport across both membranes during the cardiac cycle.

1.2.1. Role in systolic Ca^{2+} homeostasis. Depolarization of the surface membrane and t-tubules during the action potential leads to the opening of voltage-gated L-type Ca^{2+} channels (LTCCs). Within dyadic junctions, the resulting Ca^{2+} influx triggers additional Ca^{2+} release from colocalized ryanodine receptors (RyRs) in the jSR. This process, termed Ca^{2+}-induced Ca^{2+} release (CICR), underlies the systolic Ca^{2+} transient and initiation of contraction. Because this process is initiated at t-tubules, it is perhaps not surprising that t-tubule organization is an important determinant of the synchrony of Ca^{2+} homeostasis across the width of the cell (**Figure 1**). For example, healthy rat and mouse cardiac myocytes exhibit a high density of well-organized t-tubules and dyads and in turn exhibit a quite synchronous and rapid rise the Ca^{2+} transient (14, 15). This highly efficient system appears to be essential for triggering rapid and forceful



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Decreased t-tubule density decreases the synchrony of Ca^{2+} release. (*a*) Confocal line scan images showing uniform Ca^{2+} release in a rat ventricular cell consistent with a regular t-tubule network, whereas similar data from a pig ventricular myocyte show areas of delayed release corresponding with the absence of t-tubules. Panel adapted with permission from Reference 8; copyright 2014, American Journal of Physiology, and Reference 161; copyright 2002, American Heart Association. (*b*) Variability in Ca^{2+} release occurs in atrial cells where Ca^{2+} rises initially at the surface sarcolemma in cat atrial cells that lack t-tubules, slowing the central Ca^{2+} transient. T-tubules in sheep atrial cells promote a rapid rise in Ca^{2+} in the cell interior. Panel adapted with permission from Reference 7; copyright 2009, American Heart Association, and Reference 162; copyright 2003, Wiley. The synchronous rise in Ca^{2+} in rat ventricular cells is lost following detubulation (*c*) or cell culture (*d*), resulting in a smaller amplitude and slowed upstroke of the Ca^{2+} transient. Panel *c* adapted with permission from Reference 91; copyright 2004, Oxford Univ. Press, and panel *d* from Reference 163; copyright 2004, International Society of Heart Research. Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CT, central; SS, subsarcolemmal; t-tubule, transverse tubule.

myocyte contraction in these small rodents with high heart rates. Less-homogeneous Ca^{2+} release occurs in larger species, which have lower heart rates, as cardiac myocytes exhibit more gaps between t-tubules (16). In these cells, Ca^{2+} released at dyadic junctions diffuses into the gaps, where it then triggers delayed Ca^{2+} release from RyRs at these sites. Similarly, dyssynchronous Ca^{2+} release occurs in those atrial cells that have low t-tubule density (7, 8). In the most extreme case, atrial cells that are devoid of t-tubules exhibit a U-shaped pattern of Ca^{2+} release, as CICR initially triggered at the surface membrane propagates toward the cell interior as a Ca^{2+} wave between uncoupled RyRs (8, 17). However, the impact of the heterogeneous Ca^{2+} transients on atrial mechanics is more difficult to predict given the preferential expression of the fast α-myosin heavy chain isoform (18), the smaller Ca^{2+} transient amplitude, and the higher Ca^{2+}-buffering properties of atrial myocytes (19). Nevertheless, the resulting slow rising phase and the smaller Ca^{2+} transient amplitude are associated with lower force development in the atria compared with the ventricles (18, 20).

A similar Ca^{2+} release pattern can be reproduced in ventricular cardiac myocytes by experimental detubulation techniques (21, 22). In agreement with species differences in t-tubule and dyadic densities, data indicate that the proportion of orphaned RyR sites in the human heart is approximately 30% (23) but only 15% in the rat heart (24). Recent studies have indicated that the CICR process is finely tuned not just by the presence or absence of dyads but by precise positioning of Ca^{2+}-handling proteins within these structures. On the t-tubule side of the dyad, LTCCs reside within caveolae and are delivered to these sites by microtubules under the guidance of bridging integrator 1 (BIN1) (25). New data have shown that JPH2 anchors LTCCs in place (26) and, interestingly, that clustering of LTCCs may allow their functional coupling (27). Importantly, the muscle-specific caveolin isoform caveolin-3 (Cav-3), which plays a key role in forming caveolae and t-tubules, also interacts with both LTCCs and protein kinase A (PKA). This arrangement enables PKA to phosphorylate the LTCC accessory protein Rad, thereby relieving its constitutive inhibition of the channel and augmenting LTCC current (28, 29). Thus, accumulating evidence shows that LTCC localization and function within t-tubules are intimately coupled.

Bridging integrator 1 (BIN1): a protein that senses and drives membrane curvature; also known as amphiphysin II (AmphII)

Caveolin-3 (Cav-3): muscle-specific caveolin isoform involved in the formation of flask-shaped membrane structures known as caveolae

Unlike skeletal muscle, LTCCs are not directly linked to RyRs in the SR membrane in cardiac myocytes, and the relative positioning of these channels is thus less rigidly organized. Indeed, although it was previously believed that LTCCs were apposed to large, grid-like arrangements of RyRs, recent studies have indicated that RyR organization is quite variable in cardiac myocytes, with channels present in more numerous, smaller clusters (24, 30, 31). It has been proposed that neighboring RyR clusters with sufficiently short distances between them may constitute a Ca^{2+} release unit (30). In this way, Ca^{2+} influx via one or more LTCCs is suggested to elicit a Ca^{2+} spark that is cooperatively generated by several RyR clusters. Importantly, such RyR arrangements are malleable. RyRs are trafficked to dyads by BIN1 (32), and inter-RyR positioning appears to be

modified by cytosolic [Ca^{2+}] (33) and the phosphorylation status of the channels (34). Finally, another dyadic protein, JPH2, has also been shown to regulate RyR activity (35), and it is well established that RyR sensitivity is regulated by a host of post-translational modifications and binding partners, providing ample opportunity for control of fine-tuning Ca^{2+} release.

1.2.2. Role in diastolic Ca^{2+} homeostasis. Following release, Ca^{2+} is recycled into the SR by the SR Ca^{2+} ATPase (SERCA) and removed from the cell by the Na^+-Ca^{2+} exchanger (NCX) and, to a lesser extent, the plasmalemmal Ca^{2+} ATPase (PMCA). Both the NCX and PMCA are expressed at higher density within t-tubules than in the surface membrane (36, 37), indicating that t-tubules play a critical role in not only systolic but also diastolic Ca^{2+} homeostasis. Slowed removal of Ca^{2+} via the t-tubules can reduce the speed of cellular relaxation, but also the extent of relaxation, as elevated diastolic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ reduces diastolic sarcomere length. Since SERCA and NCX compete for the same pool of Ca^{2+}, reduced removal of Ca^{2+} by t-tubules can lead to increased SR Ca^{2+} content and Ca^{2+} transients and greater contractility (38, 39).

While it is established that the NCX is present along both transverse and longitudinal tubules (40), its precise localization remains unclear. However, it appears that at least a fraction of NCX proteins are located sufficiently close to RyRs to allow rapid Ca^{2+} removal during a Ca^{2+} spark (41, 42). Further evidence of functional colocalization of these proteins comes from studies showing that Ca^{2+} entry by reverse-mode NCX activity can serve as a modest trigger for RyR Ca^{2+} release (43). Although the precise distances required to support such NCX-RyR interactions remain unclear, Jayasinghe et al. (41) reported that a striking 42% of RyR clusters were within 150 nm of NCX puncta. These values closely match earlier immunogold labeling electron microscopy data reporting that NCX was within ~110 nm of the RyR (44). Notably, in this same study, the LTCC, which is strongly influenced by Ca^{2+} release from the RyR, was at least as close to the RyR as was the NCX (~140 nm using the same methodology), suggesting that at least some NCX is close enough to the RyR to influence the properties of RyR mediated Ca^{2+} release.

Importantly, the directionality of NCX function is critically set by local cytosolic Na^+ levels. Thus, augmented Na^+ influx, for example via late-Na^+ current or decreased Na^+ extrusion by the Na^+-K^+-ATPase (NKA), can slow Ca^{2+} removal by NCX (45, 46). In ventricular myocytes, it has been suggested that the α_2 NKA isoform plays a more important role than the α_1 isoform in locally regulating Na^+ levels near NCX (47). However, recent functional coupling between NKA and NCX was reported within the t-tubules of atrial cardiac myocytes, despite the exclusive expression of the α_1 isoform (48).

In summary, the function of t-tubules is dependent on their morphology and their dyadic junctions with the jSR. However, it is not only the macroscale arrangement of these membranes that determines the efficacy of CICR but also the nanoscale positioning of ion channels and exchangers on either side of the dyad. In the following sections, we examine how alterations in t-tubule structure and composition during development, adulthood, and disease impact systolic and diastolic function at the level of the cardiac myocyte and whole heart.

2. PHYSIOLOGICAL REGULATORS OF CARDIAC T-TUBULE DEVELOPMENT

T-tubules are labile structures, a physiologically advantageous adaptation allowing the heart to respond to changing demands. For example, increased t-tubule density occurs during postnatal development and following exercise (49, 50). In both cases, increased t-tubule density is associated

Esp15 homology domain protein (EHD): regulates recycling of membranes and receptors; also known as PAST1 or testilin

with augmenting magnitude and kinetics of systolic Ca^{2+} release and contractile function. These processes appear to be carefully orchestrated and, in the case of the developing heart, coincide with cellular hypertrophy, as larger cells can less efficiently rely on propagation of Ca^{2+} released at the cell surface. In contrast, reduced t-tubule density occurs in aging (51) and, as discussed in detail in subsequent sections, is a hallmark feature of many cardiac diseases where it is argued to be a significant driver of disease progression rather than a mere consequence of the disease (52). Here, there appears to be a critical mismatch between t-tubule density and cell size. Given the pathological importance of adverse t-tubule remodeling, we argue that clues as to how to reverse such deleterious remodeling can be obtained by first considering the mechanisms responsible for t-tubule formation in various healthy physiological conditions.

2.1. Paradigms Informing on the Factors Regulating T-Tubule Formation

In this section, we present an overview of how t-tubules develop postnatally and their growth during stem cell maturation. Using data from both skeletal muscle and the heart, we illustrate the important advances to our understanding of how t-tubules are built and thus enable the identification of key molecular factors underpinning t-tubule biogenesis.

2.1.1. Postnatal t-tubule development. T-tubules are absent at birth in the ventricle of small mammals and develop postnatally (10, 53) (**Figure 2**), whereas in larger species, such as sheep and humans, t-tubule development begins in utero (11, 54). It is unknown whether these differences reflect differing mechanisms of t-tubule formation between species or developmental maturity at birth. Thus, prior to t-tubule development the highly specialized organization of Ca^{2+} handling machinery required for efficient Ca^{2+} cycling in the adult myocardium is absent.

T-tubule development is thought to drive changes in excitation contraction (EC) coupling leading to an increase in the amplitude of the Ca^{2+} transient (10). Most but not all studies (55) show an increase in L-type Ca^{2+} channel current ($I_{Ca\text{-}L}$) during neonatal development (10, 56) and, importantly, LTCC coupling with RyRs is increased (57, 58). Although the SR is thought to be sparse at birth, RyRs localize to the Z-line prior to t-tubule formation (10, 59) and are augmented in line with t-tubule growth as development proceeds (60). Thus, functional dyads appear as t-tubules form early in development, and these dyads become packed with LTCCs and RyRs as development continues (53).

Early t-tubules form as irregular short invaginations that sprout in from the plasma membrane and extend toward the cell interior (10, 53, 58, 59). In skeletal muscle, multiple mechanisms have been suggested for the formation and extension of these precursor t-tubules. One hypothesis involves caveolae, flask-shaped, cholesterol-rich microdomains which, in the heart, are abundant in Cav-3. Here, Cav-3 is required for the morphogenesis of caveolae that can form interconnected networks, leading to the idea that early t-tubules arise as a result of repeated Cav-3-dependent caveolae formation (61, 62). An alternative mechanism derives from studies tracking the formation of zebrafish skeletal t-tubules. In this case, it has been proposed that the initial tubule formation uses the endocytic machinery and that the Esp15 homology domain protein (EHD) family member EHD1 plays a key role (63). Thereafter, t-tubule extension is sensitive to the disruption of trafficking pathways and may involve vesicles interacting with the forming t-tubule (63). This bears similarities to earlier work suggesting that t-tubules were formed or elongated by the addition of new membrane from the Golgi complex by exocytosis (64). Stabilization of these early t-tubules in a process termed endocytic capture may occur either via a direct interaction with the sarcomere or indirectly by interactions with the adjacent SR network, which would allow parallel maturation of the two membranes (63). The nature of this interaction remains unknown but, at

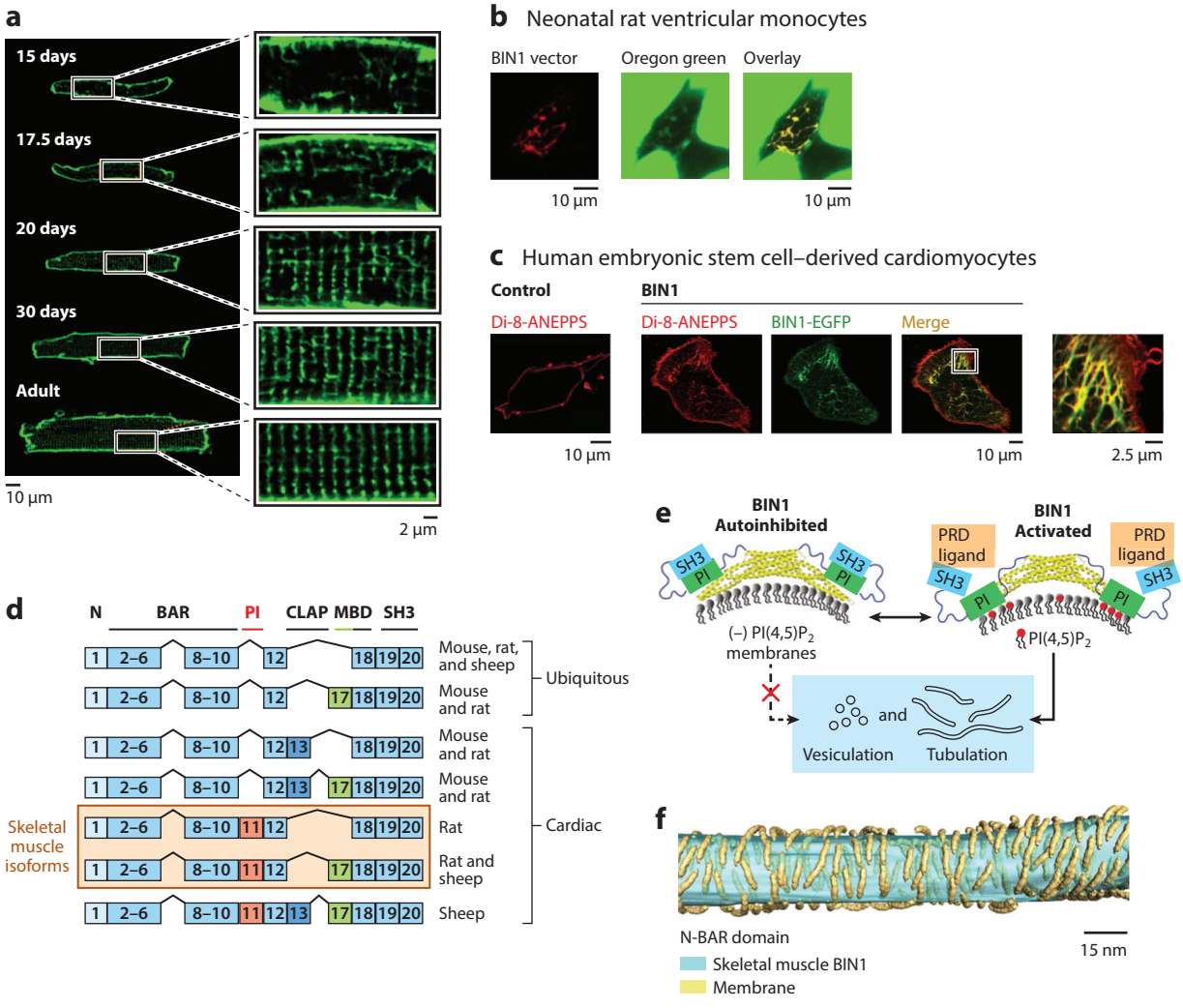


Figure 2

T-tubule development and the role of BIN1. (*a*) Confocal imaging of the developing rat ventricular t-tubule network stained with Di-8-ANEPPS at 15, 17.5, 20, and 30 days after birth. Panel adapted with permission from Reference 53; copyright 2019, Wiley. (*b*) Neonatal rat ventricular myocyte transfected with BIN1 expression vector showing tubule structures (mKate2; *red*). Extracellular Oregon green filled the tubule structures (*overlay in yellow*) showing that tubules connect to the extracellular space. Panel adapted with permission from Reference 80; copyright 2019, Springer Nature. (*c*) Human embryonic stem cell–derived cardiomyocytes in control or expressing BIN1 stained with Di-8-ANEPPS (*red*) or showing BIN1-EGFP expression (*green*) with overlay (*yellow*) of an enlarged region (*right*). Panel adapted with permission from Reference 92; copyright 2019, Wiley. (*d*) Exon maps for BIN1 isoforms found in the heart (4, 80, 95). (*e*) Autoinhibition of BIN1, where the SH3 domain interacts with the PI domain, can be relieved by PRD peptides (e.g., dynamin 2) or $PI(4,5)P_2$ to allow vesiculation and tubulation. Panel adapted with permission from Reference 100; copyright 2014, ACS Publications. (*f*) Tomographic reconstruction of the N-BAR domain of skeletal muscle BIN1 (*blue*) and membrane (*yellow*). Panel adapted with permission from Reference 98; copyright 2016, Elsevier. Abbreviations: BAR, Bin-amphiphysin-Rvs167; BIN1, bridging integrator 1; CLAP, clathrin and activator protein-binding domain; EGFP, enhanced green fluorescent protein; MBD, Myc-binding domain; PI, phosphoinositide; PRD, proline-rich domain; t-tubule, transverse tubule.

least in the heart, future work might investigate a role for JPH2 or BIN1, two proteins known to interact with membrane components on both sides of the dyad. Although less is understood about cardiac t-tubule growth, available data suggest that t-tubule elongation can occur by vesicles docking with either the surface membrane and traveling up the t-tubule or by preassembled LTCC and RyR vesicles docking directly with the t-tubule itself (65).

In early development, both skeletal and cardiac t-tubules are predominantly longitudinal rather than transverse (53, 66). In skeletal muscle it is thought that t-tubules dock with the SR and RyRs are incorporated during the longitudinal phase (66). Similar early dyadic assembly has been observed along longitudinal tubules in cardiac myocytes, although incorporation of RyRs into the dyad continues after they become transversely oriented along the Z-line (53, 66).

Whatever the precise mechanism of membrane incorporation into t-tubules, it is clear that t-tubule development requires careful membrane assembly and maintenance. At least in skeletal muscle, these processes have been suggested to critically involve the autophagy pathway (67). Autophagy is well known for its role in the degradation of cellular constituents by delivering them to lysosomes. By studying t-tubule disassembly and reassembly in *Drosophila* metamorphosis, autophagosomes were found to deliver t-tubule membrane to organelles such as lysosomes for storage and then to redeploy these components for t-tubule assembly later (67). It is unknown if autophagy contributes to membrane movement and fusion in cardiac t-tubule development, but given that the system is activated by developmental hormones in the heart, this is an attractive hypothesis (68).

2.1.2. Key proteins involved in postnatal t-tubule formation. Numerous proteins have been implicated in t-tubule biogenesis in the heart, including Cav-3, JPH2, RyR, dysferlin, and BIN1 (**Figure 3**). Although robust data highlight the importance of these proteins, deletion studies performed to date have failed to result in the complete loss of cardiac t-tubules. This highlights the potential for redundancy in mechanisms driving t-tubule formation and/or that critical players in tubulogenesis remain to be identified.

2.1.2.1. Cav-3. Cav-3 has been widely implicated in t-tubule formation. However, t-tubules are still able to develop following Cav-3 knockout (KO) in both skeletal and cardiac muscle, albeit at a lower density and with structural disorder (69, 70). The longitudinal nature of skeletal muscle t-tubules following Cav-3 KO has led to the suggestion that Cav-3 plays an important role in organizing the maturing t-tubule network (69). Finally, as the Cav-3 KO mouse lacks caveolae in skeletal and cardiac muscle (69), mechanisms other than successive caveolae formation likely also play a role in t-tubule formation.

2.1.2.2. JPH2 and RyR. The dyad-spanning protein JPH2 is important for maintaining dyadic structure and has also been implicated in t-tubule maturation during cardiac development (71, 72). JPH2 levels increase through cardiac postnatal development in some but not all studies (53, 71, 72), and JPH2 begins to appear at the z-disc as t-tubules mature (10). Knockdown of JPH2 inhibits t-tubule maturation, abolishing the developmental increase in transverse elements, while its overexpression accelerates t-tubule maturation (71, 72). Using CRISPR/Cas9-AAV9-based somatic mutagenesis, Guo et al. (73) observed that JPH2 was deleted from a fraction of cardiac myocytes (mosaicism) without causing cardiac dysfunction that could affect t-tubules independently of JPH2. Given that t-tubules could develop almost normally in the cells lacking JPH2, the authors suggested that JPH2 may serve a primary role in t-tubule maintenance rather than biogenesis (73). Interestingly, this study also identified the RyR as being important for t-tubule

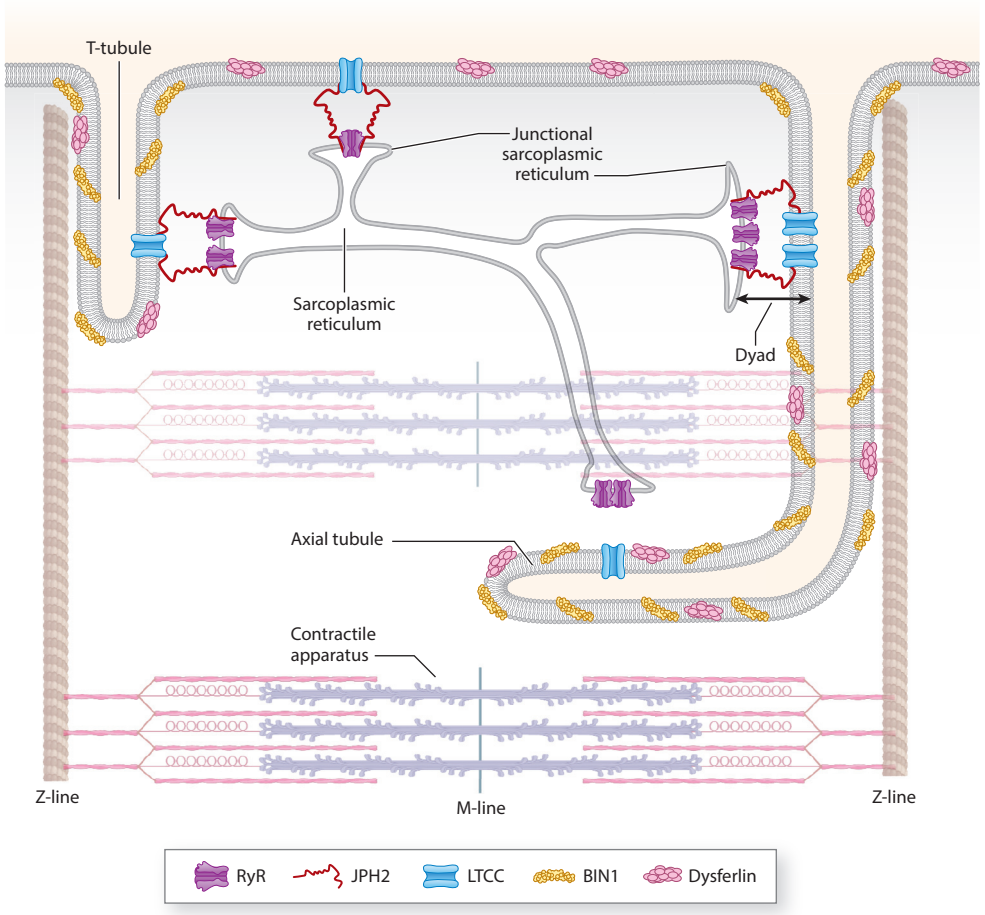


Figure 3

Putative regulators of t-tubule development and maturation. Schematic representation of the involvement of key proteins suggested to be involved in the formation of t-tubules and cardiac dyads. Dysferlin (75) and BIN1 (80, 92) can both drive de novo tubule formation. During development axially arranged tubules are more evident and lack JPH2 tethering at the dyad, leading to impaired Ca^{2+} release during systole (72, 73). Abbreviations: BIN1, bridging integrator 1; JPH2, junctophilin-2; LTCC, voltage-gated L-type Ca^{2+} channel; RyR, ryanodine receptor; t-tubule, transverse tubule.

maturation. Whether RyRs provide structural support for developing t-tubules or whether local Ca^{2+} release supports t-tubule growth remain interesting questions yet to be addressed (73).

2.1.2.3. Dysferlin. The membrane repair protein dysferlin, a member of the ferlin protein family, localizes to developing t-tubules in skeletal muscle (74). This protein can also form tubule structures in cells lacking t-tubules (75), which is in line with its suggested role in t-tubule formation (76). Dysferlin deficiency gives rise to dilated, irregularly shaped and longitudinally oriented t-tubules in skeletal muscle. Increasing colocalization between dysferlin and the LTCC during postnatal cardiac development, which, taken together with the fact that dysferlin KO results in cardiac t-tubule remodeling, suggests that dysferlin could play similar roles in the heart (74, 77).

T-tubule defects in dysferlin-deficient muscle resemble those following Cav-3 KO; intriguingly Cav-3, dysferlin, and other membrane repair proteins such as EHD1 also play important roles in vesicle trafficking, which may be relevant to t-tubule formation. In skeletal muscle, it has been suggested that dysferlin acts to fuse Cav-3-containing vesicles with developing t-tubules (78), consistent with dysferlin's localization at developing t-tubules (74). Therefore, dysferlin and Cav-3 may contribute to t-tubule biogenesis by coordinating vesicles with developing skeletal muscle t-tubules. However, it remains to be established whether a similar process occurs in the heart.

2.1.2.4. BIN1/amphiphysin 2. It is clear that the membrane curve-generating protein BIN1, also known as amphiphysin 2 (AmphII), is a key player in t-tubule biogenesis in skeletal and cardiac muscle. BIN1 can build t-tubule structures in both skeletal and cardiac muscle cells lacking these structures, and its global deletion is perinatally lethal due to cardiomyopathy (79–81). The tubulating ability of BIN1 is similar to that discussed for dysferlin; however, as tubules formed by these proteins have structural differences, it has been suggested that BIN1 and dysferlin act on distinct subelements of the t-tubule system (75). EHD1 is reported to be a negative regulator of BIN1-induced t-tubule formation, as decreased EHD1 levels (in heterozygous mice) produced ectopic and excessive t-tubule development (82, 83). It is unclear whether EHD1 also exerts effects on t-tubules due to mislocalization of Cav-3 or dysferlin (82, 83). Of note, like EHD1, BIN1 plays a role in endocytic recycling (84). However, it remains unknown whether endocytic capture features in t-tubule generation in the heart and whether BIN1 contributes to this process.

Despite a key role for BIN1 in t-tubule biogenesis, cardiac t-tubules still exist in the cardiac-specific Bin1 KO mouse (4), supporting the idea that there is no single mechanism for t-tubule formation. This research group also revealed that, in addition to shaping membrane, BIN1 provides a membrane anchor for microtubules, allowing the delivery of LTCCs to the t-tubule, where they colocalize in clusters (25). In addition, BIN1-induced microdomains play a role in the recruitment of phosphorylated RyRs into the dyad following β-adrenergic stimulation (32). Together these observations suggest that BIN1 can influence dyad structure on both the t-tubule (sarcolemmal) and SR membranes. The specific role of BIN1 in shaping the dyad has been further investigated by driving t-tubule growth with Bin1 in stem cells.

2.1.3. T-tubule growth during stem cell maturation. Stem cell maturation is a useful model for studying cardiac cell development. Indeed, numerous studies have focused on the conditions required for human-induced pluripotent stem cell–derived cardiac myocyte (hiPSC-CM) maturation, including the formation of t-tubules, a robust β-adrenergic response, and adult-like Ca^{2+} transient properties (85). Here, we discuss recent advances in our understanding of factors that encourage t-tubule development in these cells and thus the maturation of Ca^{2+} handling.

With certain stimuli, hiPSC-CMs are able to develop t-tubules even without exogenous expression of t-tubule-associated proteins (86, 87). Glucocorticoid and thyroid hormones advance maturation and growth of the fetal heart, including improvements in z-disc assembly and contraction/relaxation (88, 89). Inclusion of these hormones, along with a Matrigel substrate to provide physiological stiffness, was observed to facilitate t-tubule formation and maturation of EC coupling in hiPSC-CMs (86). This process was found to be dependent on the glucocorticoid receptor (68); the authors suggested that hormone treatment primes the cells for maturation that occurs upon proper mechanical cues derived from the substrate. The predominantly longitudinal nature of the hiPSC-CM t-tubules and accompanying synchronization of Ca^{2+} handling draw parallels to postnatal development of t-tubules and EC coupling in small rodents (86). Indeed, maturation in both cell types include a shift in BIN1 and Cav-3 localization from a perinuclear to cytosolic distribution and alignment of JPH2 along Z-lines (86). Glucocorticoids have also been shown to preserve

cardiac t-tubules by upregulating autophagic flux (68). Although this issue remains unexplored in the heart, these data are consistent with the idea that t-tubule membranes are fluid, undergo regular turnover, and agree with the concept that restricting turnover by decreasing autophagic flux adversely impacts t-tubule maintenance.

T-tubule development was also achieved in cardiac tissue formed from early-stage hiPSC-CMs by intensity training (87). Increased wall stress or tension is thought to be an important driver for cardiac development (88, 90), and it is interesting to speculate that training-engineered myocardium or culturing cells on Matrigel (86) may similarly provide key mechanical cues necessary for t-tubule development (86, 87). The loss of a mechanical driver is also consistent with t-tubule loss following the culture of isolated adult cardiac cells (91). Notably, intensity training induced t-tubules that colocalized with BIN1, RyR2, LTCCs, and functional SR stores, thus conferring responsiveness to catecholamines (87). Because newly formed t-tubules in hiPSC-CMs colocalize with BIN1 and exhibit matured Ca^{2+} handling, we might reasonably speculate that BIN1 assists with both aspects of development (25, 32, 80, 92).

Cardiac dyad: the complex formed by close apposition of LTCCs and RyRs across an ~12-nm-wide dyadic cleft

2.2. The Role of BIN1 and Its Interactome in Shaping T-Tubules

There is considerable evidence that BIN1 is crucial to t-tubule and dyadic development and may play multiple roles. Exogenous expression of BIN1 in cells lacking t-tubules [human embryonic stem cell–derived cardiomyocytes (hESC-CMs) and neonatal cells] induces a BIN1-coated, disordered t-tubule network (80, 92) (**Figure 2**). As cells mature, BIN1 expression increases, as does the length and width of tubules (92), suggesting a dual role of BIN1 in tubule formation and growth. However, it remains to be determined whether BIN1 is involved in vesicle fusion to facilitate t-tubule extension.

BIN1 interacts closely with the SR in some regions and is able to decrease SR motility as cells mature (92). Although it is unclear how BIN1 may stabilize the developing SR, we could speculate that the endocytic capture mechanism is involved. Likely, by acting as a point for microtubule-mediated delivery (25), BIN1 increases the LTCC cluster area and with it the probability of cooperative gating (92). BIN1 also promotes RyR localization to LTCCs, thereby stabilizing dyads and enhancing Ca^{2+} release (92). Thus, BIN1 appears to be centrally involved in not only membrane bending and t-tubule formation, but also the maturation and stability of the cardiac dyad as a functional unit. In the following sections, we discuss how the structural features of BIN1 enable this functionality together with its binding partners. Given the nascency in the cardiac field, this discussion draws information from work in skeletal muscle and cell lines where concepts may be common to the heart.

2.2.1. The structure and function of BIN1.

BIN1 is a member of the BAR (Bin-amphiphysin-Rvs167) domain superfamily where the N-terminal BAR domain has numerous roles, including membrane binding and the ability to sense and generate membrane curvature (93). *BIN1* is a 20-exon gene with multiple ubiquitous and tissue-specific exons (**Figure 2**). In skeletal muscle, a phosphoinositide (PI)-binding domain is necessary for t-tubule formation (79, 94).

In the heart, the role of the BIN1 PI domain is debated. Four BIN1 isoforms have been identified in the mouse, all lacking the PI domain (4). However, in the sheep and rat, the major BIN1 isoforms contain the PI domain, and exogenous expression of these isoforms drives tubulogenesis (80, 95), suggesting that the role of BIN1 and the PI domain in tubulating membranes in these species may be similar to that of skeletal muscle (**Figure 2**). There may also be species- and isoform-dependent differences in the manner by which BIN1 controls the fine structure of t-tubules. In the mouse, the BIN1+13+17 isoform is thought to underlie the formation of dense

inner membrane folds of the t-tubule network, producing a slow diffusion zone for Ca^{2+} and K^+ ions, the removal of which prolongs the action potential duration and increases ventricular arrhythmias (4). To date, t-tubule microfolds have not been reported in the normal heart of larger mammals, for example, in sheep cardiac myocytes, which lack BIN1+13+17 (80). Other BIN1 isoforms may provide roles that are shared between species. Notably, recent evidence has suggested an important role for ubiquitous BIN1 acting in the heart as a Z-line anchor for the tubule forming PI domain–containing isoforms (95). Further work is required to elucidate the role of these different BIN1 isoforms in the t-tubule lifecycle in health and disease (95).

2.2.2. Roles for the lipid environment and binding partners of BIN1. N-BAR proteins such as BIN1 have the ability to sense and generate membrane curvature, with the balance between these roles depending on their density at the membrane (96). BIN1 is thought to be targeted to the membrane by an interaction between its PI domain and phosphatidylinositols, where roles for $PI(4,5)P_2$, PI(3)P, PI(4)P, and PI(5)P have been suggested (79, 94). More recently, a role for electrostatic interactions has also been identified in regulating BIN1 membrane binding and membrane bending (97). This is consistent with work demonstrating that BIN1 tubulation depends on electrostatic attraction to $PI(4,5)P_2$ and not necessarily the PI domain per se (98). Therefore, tubulation could be controlled by altering the membrane charge and recruitment of BIN1 to the membrane (97) and the PI domain could enhance binding (79).

BIN1's ability to sense and generate membrane curvature is thought to be autoinhibited by the C-terminal SH3 domain binding to the PI domain and inducing a conformational change (99, 100) (**Figure 2**). The presence of $PI(4,5)P_2$ in the cell membrane, or the binding of dynamin, a large GTPase, to the SH3 domain is thought to remove this autoinhibitory effect, thus allowing BIN1 to sense and generate membrane curvature (100). The recruitment of dynamin to the membrane is thought to rely on the BIN1-dependent clustering of PIs and, because BIN1 appears to increase upon dynamin binding, a feedback loop between the two proteins has been suggested (101). Indeed, mutations of dynamin are associated with centronuclear myopathy (characterized by weakness and wasting of skeletal muscle), and t-tubule disorganization may play a role but through yet-to-be-established mechanisms (102).

Mutations in an additional BIN1 binding partner, the lipid phosphatase myotubularin (MTM1), cause another type of centronuclear myopathy. MTM1 also enzymatically enhances BIN1 membrane tubulating capacity (103). BIN1 and MTM1 may functionally compensate for one another, as increasing BIN1 is able to decrease the myopathic phenotype in MTM1 myopathies (104, 105). However, the precise mechanism by which MTM1 alters tubulation is unknown, but it could include altered interactions with BIN1 and dynamin or via changes in the lipid environment (94, 106).

In summary, while it is clear that BIN1 is important for shaping both the t-tubule and the dyad, the precise mechanisms are not fully understood. In the heart, research has begun to shed light on the ability of BIN1 to multitask; however, it is not yet clear if parallels can be drawn with mechanisms of tubulation described in skeletal muscle. Only recently have we begun to unfold the importance of BIN1 in the heart, and future research undoubtably has much to reveal.

3. T-TUBULE CHANGES IN DISEASE, THE INTERRELATED IMPACT ON DYADIC STRUCTURE, AND OPPORTUNITIES FOR THERAPY

Given the preceding discussion, it follows that any changes to the properties of t-tubules in disease will lead to perturbed cellular Ca^{2+} homeostasis. In this regard, there is near universal agreement that t-tubule remodeling is a key finding across diverse cardiac diseases spanning preclinical

models, human samples, and both the atria and ventricles (**Supplemental Table 1**). The major observations with respect to t-tubule remodeling in disease include a reduction in density, patchy distribution, and disorganization with a greater fraction of longitudinal (axial or lateral) t-tubules. However, there is an important caveat in that in several recent studies examining t-tubule remodeling in heart failure with preserved ejection fraction (HFpEF), the t-tubule network density is either unaltered (107, 108) or increased; lateralization is not a feature until diastolic dysfunction is severe in human HFpEF (108). Irrespective of the underlying etiology and impact on ejection fraction, t-tubule remodeling is associated with dysfunctional intracellular Ca^{2+} homeostasis. Exemplar findings where t-tubule properties and cellular function have been investigated simultaneously include reduced Ca^{2+} transient amplitude with increased dyssynchrony of Ca^{2+} release (14, 16, 23). Of note, the extent of t-tubule remodeling is correlated with impaired cardiac function and wall stress (109, 110). Importantly, ex vivo simulation of high wall stress conditions can promote a reduction in t-tubule density (111). Notwithstanding the clinical importance of HFpEF as a clinical entity, there remains a relative paucity of preclinical models that are fully representative of this condition and, as such, the focus of the remainder of this section is primarily on aspects of ventricular t-tubule remodeling in systolic heart failure [heart failure with reduced ejection fraction (HFrEF)].

Supplemental Material >

Heart failure: a disease of the heart leading to impaired cardiac function (contraction and/or relaxation)

There still remains the chicken and egg question: Does t-tubule remodeling drive heart failure progression or is it a consequence of heart failure? Although this has not been extensively studied, some evidence shows that the changes in t-tubule distribution and density occur before the onset of heart failure symptoms (52, 80). Further supporting the developing hypothesis that t-tubule remodeling is a driver of cardiac dysfunction, cardiac resynchronization therapy (112), phosphodiesterase 5 inhibition (80, 113), and mechanical unloading (114) are capable of reversing t-tubule remodeling in heart failure, and the normalization of t-tubule distribution and density in these studies is associated with improvements in contractile function and systolic Ca^{2+}. Such plasticity of the t-tubule network was also noted in the earlier sections discussing development, cell culture, and exercise, and we propose that these paradigms provide an invaluable tool with which to understand the factors regulating t-tubule synthesis, stability, and degradation. Henceforth, we examine those previously identified factors in relation to heart failure and as potential therapeutic interventions to target t-tubule restoration. However, we first consider the mechanisms whereby t-tubule disruption causes alterations to systolic Ca^{2+} in heart failure.

3.1. How Does T-Tubule Remodeling in Heart Failure Drive Altered Cellular Ca^{2+} Homeostasis?

In the following section we consider how changes in t-tubule geometry in disease impact dyadic structure and systolic Ca^{2+}.

3.1.1. Dyadic reorganization. Some of the earliest work demonstrating the functional importance of the ventricular t-tubule network in regulating cardiac Ca^{2+} fluxes and systolic Ca^{2+} was provided by Orchard and colleagues (21, 115), who developed the technique of osmotically shocking cardiac myocytes with formamide to cause chemical detubulation (**Figure 1**). Here, in rat ventricular myocytes, formamide treatment was associated with a decrease in membrane capacitance of ~25%. Complementing these initial studies, recent work has shown that the tricyclic antidepressant imipramine produces a more complete detubulation resulting in a ~40% decrease in membrane capacitance (22), which is more in line with previous calculations of the fraction of the cell membrane present within the t-tubule network (2). Irrespective of the method of detubulation, the principal finding from these and similar studies is that, in so-called healthy

myocytes, LTCCs are concentrated to the t-tubule membrane, and following detubulation the systolic Ca^{2+} transient is smaller and heterogeneous, propagating as a Ca^{2+} wave from the cell periphery to the cell interior. The well-characterized changes to the t-tubule network in heart failure would therefore be predicted to produce the observed changes in the spatiotemporal properties of the systolic Ca^{2+} transient reported in heart failure.

At the cellular level, the loss of t-tubules in heart failure leads to decoupling of the LTCCs that are concentrated along the t-tubule membrane and their normally closely apposed SR Ca^{2+} release channels (RyRs), in what has been coined dyadic orphaning (15). Based on acute detubulation studies, such orphaning provides a clear mechanism to drive alterations to the systolic Ca^{2+} transient in heart failure. However, t-tubule loss is incomplete in failing ventricular cells, with t-tubule density decreasing between 18 and 32% (108, 116, 117). Where t-tubules remain present, decoupling from the jSR (i.e., RyRs) via widening of the dyadic cleft or reduced linear apposition between the jSR (i.e., RyR) and t-tubule membrane (i.e., LTCC) has been reported (118) and proposed experimentally (119) to cause a reduction in Ca^{2+} transient amplitude. Additionally, accumulating evidence now indicates that a variety of changes occur in key dyadic components in heart failure. Focusing on the RyR, these include alterations to cluster size, cluster separation, intracluster packing density, and phosphorylation (112, 120–122). In vitro work (123) strongly supports the concept that changes to the organization of RyR clusters will alter the fidelity of Ca^{2+} release from the SR during systole. While changes in the dyadic cleft dimension or RyR cluster properties are, on first appearance, a plausible mechanism further underpinning impairment of the systolic Ca^{2+} transient, the reader is referred to previous work where it is argued that such changes occurring in isolation may not have a maintained effect on systolic Ca^{2+} due to compensatory changes of SR Ca^{2+} content (124). As such, these alterations should always be viewed in the context of other molecular and functional changes occurring in heart failure, such as reduced SR Ca^{2+} uptake, augmented Ca^{2+} efflux via the NCX, and reductions in the Ca^{2+} entry via $I_{Ca\text{-}L}$ (125, 126) before assigning causality to changes in systolic Ca^{2+} or contractility.

3.1.2. Failed action potential propagation and impaired solute diffusion. T-tubule remodeling in heart failure is highly variable not only between models and different cells within the same heart, but it is also strikingly present within an individual myocyte with dilatation (varicosities), branching, constrictions, tortuosity, and inner membrane folding noted (3, 121, 127, 128) (**Figure 4**). It seems improbable that such diversity would be without functional consequence. Confirmation to this effect was demonstrated by Crocini et al. (129), who noted failed action potential propagation on some t-tubules in heart failure cells that led to a delayed rise of the systolic Ca^{2+} transient in a rat model of myocardial infarction. It appears that the changes to the physical dimensions of t-tubules, especially constrictions, varicosities, and tortuosity, can directly impair solute diffusion and impinge on action potential propagation (5, 130–132).

3.1.3. Cyclic nucleotide second messenger signaling cascades. The cardiac β-adrenergic response is substantially attenuated in heart failure (126, 133). Indeed, extensive work has identified perturbed function at each step along the classical catecholamine signaling pathway, with alterations in receptor abundance, agonist sensitivity, adenylate cyclase, protein kinase A, and phosphodiesterase, as well as protein phosphatase activity, culminating in impaired target site phosphorylation (reviewed in 134). Hereafter, we consider more specifically the impact of t-tubule remodeling on this pathway.

By combining the use of Förster resonance energy transfer (FRET) biosensors to assess cellular cAMP content and scanning ion conductance microscopy (SICM) to locally activate the β_1- and β_2-adrenergic pathways, Nikolaev et al. (133) elegantly demonstrated that β_2-adrenergic

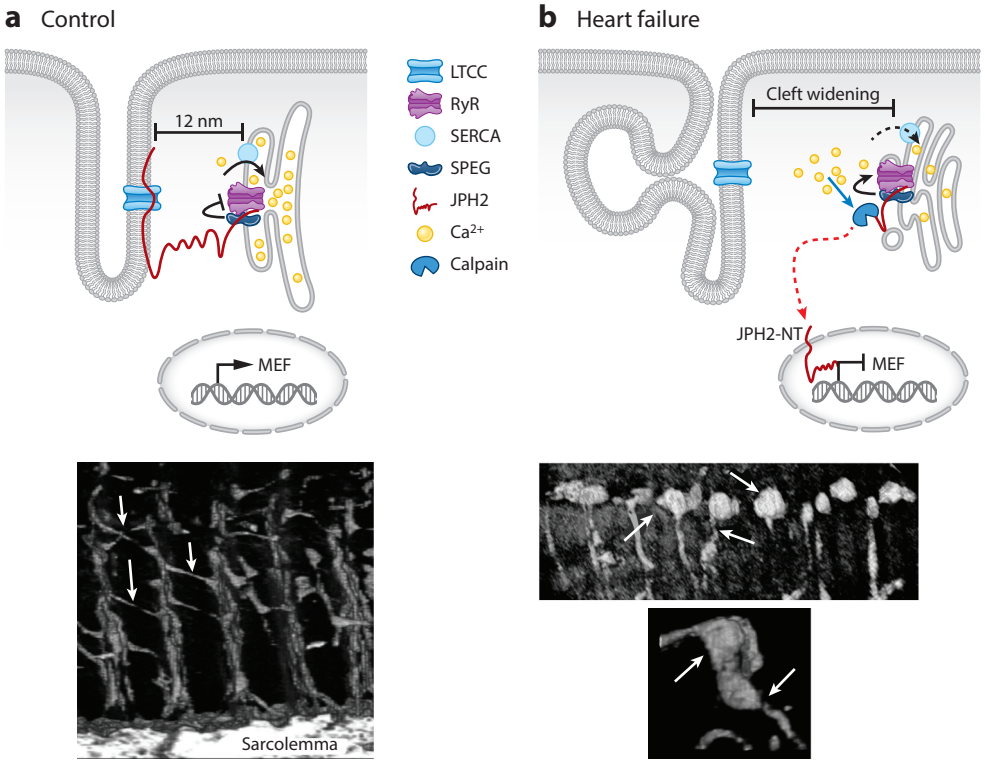


Figure 4

Dyadic architecture is maintained by the JPH2 interactome. (*a*) Schematic representation of the healthy dyad (*top*) and reconstructed ventricular t-tubules in a healthy sheep (*bottom*). In the healthy ventricular myocardium, the dyad consists of closely apposed LTCCs and RyRs that are held in close proximity by the tethering protein JPH2. The kinase SPEG interacts with and phosphorylates SERCA and the RyR to increase SR Ca^{2+} uptake and reduce RyR leak, thus keeping cleft calcium levels low; it also maintains the structural integrity of JPH2, the dyad, and the t-tubule. In the lower panel, serial block face scanning electron microscopy was used to study the structure of t-tubules in sheep ventricular myocytes. The 3D reconstruction shows regularly arranged t-tubules with branches (*white arrows*) between t-tubules at adjacent Z-lines. (*b*) In heart failure there is a reduction in SPEG kinase activity leading to increased RyR calcium leak and reduced SERCA-mediated calcium uptake (*black arrow*). The resulting elevation of dyadic calcium concentration activates calpain-mediated cleavage of JPH2 (*blue arrow*). This causes dyadic instability and is associated with cleft widening, vacuolation of the SR (*black dotted arrow*), and t-tubule conformational changes. The JPH2-NT translocates to the nucleus and thus acts as a repressor of transcription of prohypertrophic genes (*red dotted arrow*). The lower panel shows 3D reconstructions of ventricular t-tubules from a sheep heart failure model. Note the varicosities and t-tubule narrowing (*white arrows*). Lower panel images adapted with permission from Reference 3; copyright 2013, American Heart Association. Abbreviations: 3D, three-dimensional; JPH2, junctophilin-2; JPH2-NT, N-terminal fragment of JPH2; LTCC, voltage-gated L-type Ca^{2+} channel; MEF, myocyte enhancer factor; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; SPEG, striated muscle preferentially expressed protein kinase; SR, sarcoplasmic reticulum; t-tubule, transverse tubule.

receptor function was distributed away from the t-tubule to the sarcolemmal crest in heart failure, while the cell-wide change in cAMP content after β_1 selective stimulation was unaltered. The same group also demonstrated that both the β_3-adrenergic receptor–mediated nitric oxide (NO)/soluble guanylate cyclase cGMP (sGC/cGMP) (135) and natriuretic peptide receptor A

(NPRA or NPR1) particulate guanylate cyclase cGMP (pGC/cGMP) (136) signaling cascades are also concentrated at the t-tubule in the healthy myocardium. Similar to the β_2-adrenergic receptor cascade, the β_3-adrenergic receptor cascade is also redistributed from the t-tubule to the sarcolemmal crest in heart failure (135).

Although convincing, there are some potential methodological constraints associated with these classical FRET experiments that the reader should bear in mind: (*a*) Cells were maintained in culture for ~48 h to allow FRET transgene expression, (*b*) there are species differences in phosphodiesterase activities in the healthy and diseased heart (reviewed in 137), (*c*) the FRET sensors used reported global changes in cyclic nucleotides, and (*d*) there is considerable cross talk between the cAMP and cGMP cascades mediated primarily by cGMP-dependent regulation of phosphodiesterases 2 and 3 (PDE2 and PDE3) cAMP hydrolytic activity. Potential issues regarding the impact of culture on t-tubule properties were highlighted in earlier sections of this review; however, reassuringly, in a transgenic cGMP reporter mouse, changes in cGMP regulation were noted in freshly isolated hypertrophic cells (138). Because the FRET sensors used in these studies reported global nucleotide changes, the reader should interpret the findings mindful of the rapidly expanding evidence that cyclic nucleotides, exchange proteins activated by cAMP, A-kinase binding proteins, and the phosphodiesterase isoenzymes and their target proteins are not free to roam throughout the cell. Rather, accumulating evidence has indicated that these signaling molecules often exist as macromolecular complexes, thus conferring subcellular localization and specificity of effect on target proteins (for a review, see 139).

In summary, the impact of t-tubule remodeling in heart failure on the regulation of systolic Ca^{2+} is far more complex than the simple loss of coupling between LTCCs and RyRs. Although many technical limitations need to be overcome, it is evident that the t-tubule is a signaling nexus that finely tunes the response of the heart to classical inotropic modulators. The recent development of FRET sensors tuned to precise subcellular domains (140) is likely to dramatically improve our understanding in this respect.

3.2. Regulators of T-Tubules and Dyadic Structure in Heart Failure

As previously highlighted, an increasing number of proteins have been proposed as regulators of t-tubule formation and the organization of key Ca^{2+} regulatory proteins at the dyad. Here, we focus on some emergent additional factors that could contribute to the pathological remodeling of the t-tubule and dyadic structure in heart failure and address some problematic mechanistic disparities arising from earlier studies.

3.2.1. Striated muscle preferentially expressed protein kinase and junctophilin-2. The role of JPH2 in heart failure is somewhat controversial, with some studies noting a decrease (e.g., 52, 128) and others no change (e.g., 117) in JPH2 transcript or protein levels. The differences in JPH2 levels do not appear to be explicable by differing extents of cardiac dysfunction (52, 117). However, we do not exclude a potentially significant role for JPH2 in heart failure, and these discrepancies may be resolved by work exploring three additional hypotheses on the function of JPH2:

1. JPH2 is a target for phosphorylation by striated muscle preferentially expressed protein kinase (SPEG), a serine/threonine protein kinase that is exclusively coprecipitated with both JPH2 and the RyR (141). In this paradigm, SPEG phosphorylates JPH2 and impaired JPH2 phosphorylation rather than altered JPH2 abundance drives t-tubule disruption and Ca^{2+} dysregulation in heart failure (141). Furthermore, SPEG can phosphorylate both RyR at serine (Ser)^{2367}, reducing open probability (142), and SERCA at threonine (Thr)^{484},

accelerating Ca^{2+} uptake into the SR (143). As such, any disease-mediated changes in SPEG alter Ca^{2+} homeostatic mechanisms and may thereby compound any deleterious JPH2 phosphorylation-dependent t-tubule effects in heart failure.

2. Calpain mediated cleavage of an N-terminal fragment (JPH2-NT) (144, 145) that translocates to the nucleus and acts as a repressor of transcription of myocyte enhancer factor 2 (MEF2)-regulated genes. Notably, (*a*) blockade of MEF2 transcriptional regulation can prevent hypertrophy from developing or reverse established cardiac hypertrophy (146), and (*b*) *SERCA2a* gene therapy attenuates calpain-mediated JPH2 cleavage, improving t-tubule organization in an ischemia reperfusion model (147).
3. JPH2 also directly interacts with the LTCC at the α_{1C} and β_2 subunits, stabilizing the dyadic alignment of the LTCC and RyR (26). Critically, the region of JPH2 joining the LTCC is contained within the calpain-truncated, N-terminal part of JPH2. Therefore, any protective effect gained by repression of the hypertrophic transcriptome may be offset by disturbance of dyadic architecture when JPH2 is cleaved (**Figure 4**).

3.2.2. Nexilin. A relatively new addition to the battalion of proteins controlling dyadic and t-tubule architecture is nexilin, an actin-binding z-disc protein, where mutations and gene KO are associated with cardiomyopathies in human and animal models (148, 149). Somewhat like SPEG, nexilin associates with tRyR and JPH2 (149) but not LTCC subunits, and the loss of nexilin leads to a reduction in t-tubule density and an increase in Ca^{2+} sparks. However, whether degradation of t-tubule structure is a direct result of the loss of nexilin in these models or through the JPH2 interactome remains to be established. It may be that, as is the case for receptor accessory protein 5 (150), nexilin's main role is to stabilize the SR next to the RyR and t-tubule membrane, thus ensuring the close apposition of the key dyadic components and junctional SR and enabling efficient Ca^{2+} release during systole (**Figure 4**).

3.3. Opportunities for T-Tubule-Targeted Therapies in Heart Failure

Simply considering those proteins that we have already highlighted and their regulators of transcription, e.g., micro RNA-24 (miRNA-24) (151), a potentially large number of novel targets for future therapies are directed at reversing the ultrastructural remodeling and thence contractile dysfunction that occurs in heart failure, some of which have already shown preclinical effectiveness (152, 153). Equally, these same proteins may have utility as biomarkers for disease staging, as has already been demonstrated for BIN1 (154). As noted previously, given the interplay between t-tubules and systolic Ca^{2+}, the restoration of one may facilitate recovery of the other; it may be that t-tubule restoration is driven by the normalization of systolic Ca^{2+}. Given the progressive nature of heart failure, initial remodeling may be small, localized, and difficult to detect experimentally, meaning it is difficult to understand what comes first. However, t-tubule remodeling has been shown to precede echocardiographic dysfunction, suggesting that it promotes the transition from compensated hypertrophy to failure at the level of the whole heart (52).

Although there is merit in exploring these proteins and associated regulatory pathways as next-generation therapies, it is worth highlighting that several already-used therapies for heart failure and other clinical conditions can attenuate t-tubule remodeling and, of greater clinical significance, restore t-tubule density in heart failure. These include β-blockers (155), T_3-thyroxine (156), and PDE5 inhibitors (80, 113). Additionally, mechanical unloading of the heart achieved through cardiac resynchronization therapy (157), heterotopic transplantation to the low-pressure venous system (114), or left ventricular assist device (LVAD) implantation (127) have shown that it is possible to reverse remodel t-tubules in heart failure. However, the effect of unloading may

be dependent on the extent of t-tubule remodeling at the time of LVAD implantation (127). Complementing the effect of mechanical unloading on t-tubule density, endurance exercise training also attenuates reductions in t-tubule density postmyocardial infarction (50). However, further work is required to determine whether the effects of exercise on t-tubule density in heart disease are due to an attenuation of loss or de novo restoration. Furthermore, it will be instructive for the development of future targeted therapies to determine whether the exercise-mediated effects on t-tubules are due to the well-characterized, exercise-dependent reduction in peripheral resistance and blood pressure (158, 159) or other factors. Although these studies lend support to the beneficial effects of cardiac unloading being linked to protection of subcellular structure, they also highlight a growing appreciation that mechanical overload is a direct promotor of t-tubule disruption and thus heart failure. In this way, a vicious cycle is envisioned, as t-tubule loss further weakens contraction, and resulting activation of the renin-angiotensin system further increases cardiac workload. Thus, future therapeutic strategies may be aimed at unraveling and uncoupling the mechanosensory pathways that lead to t-tubule degradation in this disease.

One important note of caution with which to conclude is that while t-tubule density may be restored after heart failure is established using several of these highlighted approaches, the organization of the t-tubule network is not necessarily normal (80, 157). The implication here is that there will still be misalignment between dyadic components that may result in RyR orphaning. In some studies (15) but not all (160), RyR orphaning leads to the formation of dyssynchronous Ca^{2+} sparks, and thus potentially arrhythmias. These seemingly opposing observations may readily be resolved in future studies applying live-cell superresolution imaging microscopy approaches, where functional Ca^{2+} imaging and the nanoscale organization of the dyadic components can be achieved simultaneously.

4. CONCLUSION

Throughout this review we have attempted to highlight the functional significance of the t-tubule network in the heart and identify some of the known key factors that regulate the t-tubule network. We also discussed the nature and implications of t-tubule remodeling in the setting of heart failure and how these could be targeted in the future. From our current understanding it is clear that a large number of questions remain unresolved in this nascent field. Key among these are the identity of any master t-tubule regulator and a unifying hypothesis for why t-tubules and their associated dyadic structures become disordered in cardiac disease. We suggest that addressing these challenges will require application of a multidisciplinary mindset incorporating the latest omics methodologies, advanced correlative superresolution imaging approaches, the targeting of specific genes and protein–protein interactions, chemical and physical engineering, and ultimately clinical studies. To quote Plato and reflect on our current understanding, "the beginning is the most important part of any work."

SUMMARY POINTS

1. Transverse (t-) tubules are key to the synchronous rise of intracellular calcium in cardiac muscle, and this is achieved through concentration of the excitation contraction coupling machinery along their length.
2. T-tubules are highly plastic structures that develop after birth, respond to changes in their physical environment, and become disordered in a wide variety of cardiac diseases.

3. T-tubule disorder in all its forms has significant (adverse) effects on cardiac excitation-contraction coupling.
4. An expanding number of protein-coding genes are implicated in the regulation of the t-tubule lifecycle, but no single critical gene or protein has thus far emerged.

FUTURE ISSUES

1. What is the role of the different BIN1 isoforms and the BIN1 interactome in regulating the t-tubule lifecycle in health and in response to disease?
2. Progress is required on the identification of drugs or small molecules that can finely tune t-tubule architecture and restore normal dyadic structure in disease.
3. How do developmental or disease-mediated changes in t-tubule organization impact the compartmentalization of cyclic nucleotide second messenger pathways, and how does this affect the regulation of systolic Ca^{2+} in heart disease?
4. What are the mechanisms by which current heart failure therapies reverse t-tubule remodeling in heart failure? Does this involve relief of mechanical stress?
5. Simultaneous correlative functional and structural studies are required to fully elucidate the role of the t-tubule and cardiac dyadic remodeling in disease.

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