Dependence of Cardiac Transverse Tubules on the BAR Domain Protein Amphiphysin II (BIN-1)


Rationale: Transverse tubules (t-tubules) regulate cardiac excitation–contraction coupling and exhibit interchamber and interspecies differences in expression. In cardiac disease, t-tubule loss occurs and affects the systolic calcium transient. However, the mechanisms controlling t-tubule maintenance and whether these factors differ between species, cardiac chambers, and in a disease setting remain unclear.

Objective: To determine the role of the Bin/Amphiphysin/Rvs domain protein amphiphysin II (AmpII) in regulating t-tubule maintenance and the systolic calcium transient.

Methods and Results: T-tubule density was assessed by di-4-ANEPPS, FM4-64 or WGA staining using confocal microscopy. In rat, ferret, and sheep hearts t-tubule density and AmpII protein levels were lower in the atrium than in the ventricle. Heart failure (HF) was induced in sheep using right ventricular tachypacing and ferrets by ascending aortic coarctation. In both HF models, AmpII protein and t-tubule density were decreased in the ventricles. In the sheep, atrial t-tubules were also lost in HF and AmpII levels decreased. Conversely, junctophilin 2 levels did not show interchamber differences in the rat and ferret nor did they change in HF in the sheep or ferret. In addition, in rat atrial and sheep HF atrial cells where t-tubules were absent, junctophilin 2 had sarcomeric intracellular distribution. Small interfering RNA–induced knockdown of AmpII protein reduced t-tubule density, calcium transient amplitude, and the synchrony of the systolic calcium transient.

Conclusions: AmpII is intricately involved in t-tubule maintenance. Reducing AmpII protein decreases t-tubule density, reduces the amplitude, and increases the heterogeneity of the systolic calcium transient.

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Key Words: calcium ■ heart failure

The synchronous rise of the systolic Ca\(^{2+}\) transient in mammalian ventricular myocytes requires the presence of an extensive and regular transverse (t-)tubular system.\(^1\) These t-tubules ensure close apposition of L-type Ca\(^{2+}\) channels (LTCCs) and sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channels (ryanodine receptors [RyRs]) forming dyads or couplons where excitation–contraction coupling commences.\(^2,3\) The t-tubules are also surrounded by a continuous network of SR, which is thought to assist with amplification of the initial Ca\(^{2+}\) entry during the action potential and contribute to the synchronous rise of systolic Ca\(^{2+}\).\(^4,5\) The t-tubule and SR networks are, however, labile with disorganization and loss commonly observed in heart failure (HF).\(^5,9\) In such circumstances the loss of t-tubules leads to dysynchronous Ca\(^{2+}\) release patterns, a smaller systolic Ca\(^{2+}\) transient, and altered β-adrenergic (β-adrenergic receptor) signaling.\(^6–10\) Conversely, recovery from HF is associated with restoration of the t-tubule network along with normalization of β-adrenergic receptor signaling and resynchronization of the systolic Ca\(^{2+}\) transient.\(^9,11\)

In This Issue, see p 961

More extensive differences in t-tubule organization and density than those occurring in the ventricle during HF are known to exist between the atrium and the ventricle. For example, small mammals (mouse, rat, rabbit, etc) completely lack or possess only a rudimentary, predominantly axially arranged, t-tubule network.\(^2,14\) Conversely, some studies have suggested that limited numbers of atrial cells from smaller laboratory species such as the rat have a more ventricular-like t-tubule pattern,\(^12\) although these particular cells may be of different lineage and a feature of the pulmonary vein sleeve region.\(^16\) The poorly developed t-tubule network in these atrial myocytes leads to the characteristic early peripheral and delayed central
Ca\textsuperscript{2+} transient.\textsuperscript{12,17} More recently, however, a well-developed t-tubule system has been noted in atrial myocytes of larger species including man.\textsuperscript{18–20} Although remaining less extensive than in the corresponding ventricle,\textsuperscript{19} the t-tubule system in atrial myocytes of these larger species substantially reduces the spatial heterogeneity of the systolic Ca\textsuperscript{2+} transient.\textsuperscript{19} Moreover, as in the ventricle, the atrial t-tubule network is disrupted in HF and atrial fibrillation resulting in increased Ca\textsuperscript{2+} transient heterogeneity and dyssynchronous Ca\textsuperscript{2+} release.\textsuperscript{18,19}

Several proteins have been implicated in the biogenesis and maintenance of t-tubules including titin cap protein (telethonin), junctophilin 2 (JPH2), and the Bin/Amphiphysin/Rvs domain protein amphiphysin II (AmpII or BIN-1).\textsuperscript{21,22} Of these, the Bin/Amphiphysin/Rvs domain proteins are ubiquitously expressed, highly conserved in eukaryotes and have pleiotropic roles including sensing membrane curvature, endocytosis, and regulation of actin filament function.\textsuperscript{23,24} Certain splice variants of amphiphysin, AmpII (BIN-1),\textsuperscript{25–27} seem not to be involved in the formation of clathrin-coated vesicles and endocytosis.\textsuperscript{28,29} They are, however, highly expressed in striated muscles, localize to t-tubules, and gene deletion leads to fatal perinatal cardiomyopathy.\textsuperscript{26,30} More recently, these BIN-1 splice variants have been identified, in the mouse heart, as being involved in both t-tubule formation and causing extensive folding of the inner t-tubule membrane.\textsuperscript{31} However, the lack of a densely folded inner t-tubule membrane in other species, for example, sheep and rat as used in the present study,\textsuperscript{5} suggests that the particular BIN-1 (AmpII) splice variants responsible may be a feature of the murine myocardium.

Mutations in AmpII also lead to the inherited condition centronuclear myopathy,\textsuperscript{31} which is characterized by a severe cardiomyopathy, myopathy disarray,\textsuperscript{32} and arrhythmias.\textsuperscript{33} Moreover Drosophila AmpII mutants show flight muscle t-tubule disarray that is reversed by AmpII cDNA transfection.\textsuperscript{29} Finally, AmpII transfection induces tubule formation in Chinese hamster ovary (CHO) and the liver hepatocellular carcinoma cell line (HepG2), cell types that do not ordinarily possess t-tubules.\textsuperscript{26,34,35} and, using the 13+17 splice variant, causes t-tubule rescue in cultured BIN-1 (AmpII) heterozygous knockout cardiac myocytes.\textsuperscript{27}

In cardiac muscle, AmpII protein levels are decreased in HF, which as noted above is associated with t-tubule loss and disorganization.\textsuperscript{12,25} In addition, AmpII directs LTCC expression to the t-tubule\textsuperscript{26} and thereby provides a potential link between t-tubule loss and the decrease in L-type Ca\textsuperscript{2+} current observed in some models of HF.\textsuperscript{25,36} In the recently developed BIN-1 knockout mouse model, the heterozygote shows a decreased intensity of t-tubule staining although t-tubules in the ventricle seem to be localized correctly. However, no information was presented on how such perturbations in BIN-1 (AmpII) levels affect cellular Ca\textsuperscript{2+} homeostasis or interchamber differences in t-tubule density. This study therefore sought to determine (1) whether interchamber differences in t-tubule density are related to AmpII protein levels, (2) whether AmpII protein levels and t-tubule density change in HF, (3) whether AmpII gene silencing reduces t-tubule density, and (4) how AmpII gene silencing influences the synchronicity of the systolic Ca\textsuperscript{2+} transient. We demonstrate that (1) the amount of AmpII protein is lower in the atria, (2) AmpII protein levels and t-tubule density are lower in HF, (3) transfection of adult rat ventricular myocytes with small interfering RNA (siRNA) decreases both AmpII protein levels and t-tubule density, and (4) loss of AmpII increases heterogeneity (dyssynchrony) of the systolic Ca\textsuperscript{2+} transient. We conclude therefore that AmpII is required for the maintenance of t-tubules in cardiac muscle, and loss of AmpII is responsible for t-tubule disruption and increases the heterogeneity of the systolic Ca\textsuperscript{2+} transient. In contrast, using the same experimental approaches, we show that JPH2 is present, with sarcomeric intracellular distribution, in rat atrial and sheep HF atrial cells (which lack t-tubules) and also that JPH2 has an important role in determining t-tubule orientation rather than the overall density of t-tubules.

### Methods

A detailed Methods section is available in the Online Data Supplement.

All procedures involving animals accord to The United Kingdom, Animals (Scientific Procedures) Act of 1986 and have been approved by The University of Manchester Ethical Review Board.

### Myocyte Isolation, T-Tubule Quantification, and Animal Models

Single cardiac myocytes were isolated from the left ventricle and left atria of sheep and rats using collagenase and protease digestion methods described in detail previously.\textsuperscript{19,36–38} Myocytes and paraformaldehyde-fixed tissue sections (ferret) were stained with the voltage-sensitive aminonapthylenylpyridium dye di-4-ANEPPS, the styryl dye N-(3-trethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64), or Alexa Fluor 488–conjugated wheat germ agglutinin (WGA) and imaged confocally on a Leica SP2 microscope to visualize t-tubules, which were then quantified after image processing as described previously.\textsuperscript{19,39} HF was induced using either right ventricular tachypacing in the sheep or ascending aortic coarctation in the ferret.\textsuperscript{40,41}

### Myocyte Culture, siRNA-Mediated Gene Silencing and [Ca\textsuperscript{2+}]\textsubscript{i} Measurements

Single rat ventricular myocytes were maintained in myocyte growth medium (Promocell, UK) and transfected following manufacturer’s recommendations (Santa Cruz, USA) with 10 μmol/L siRNA (Sigma Mission siRNA or Santa Cruz scrambled siRNA) targeting AmpII, JPH2, or a scrambled (control) siRNA. Cells were transfected for 24 hours and then imaged as described above or processed for immunoblotting or immunolabeling. Changes in [Ca\textsuperscript{2+}]\textsubscript{i} were monitored using Fluor-3 acetoxymethyl ester (AM)–loaded cells on a Leica SP2 confocal microscope.

### Statistics

All data are presented as mean ± SEM from n observations/N experiments. To account for multiple observations (n) from the same animal (N) linear mixed modeling was performed (SPSS Statistics; IBM, USA). Where multiple observations or technical replicates were not...
performed, Student $t$ test, paired $t$ test, or Mann–Whitney rank-sum tests (used when the data was neither normally distributed nor had unequal variance) were used. Pearson coefficient was performed on paired data points using GraphPad Prism 5.0. Data were considered significant when $P<0.05$.

**Results**

**Interchamber Differences in T-Tubule Density and AmpII or BIN-1 Expression**

The presence of t-tubules was examined in the ventricles and atria of the rat, ferret, and sheep (Figure 1A). In agreement with previous work, a well-developed t-tubule network was found in all of the ventricular samples studied. In the rat atrium, we found essentially no t-tubules, whereas in the ferret atrium a rudimentary t-tubule network was present in some cells (Figure 1Ab). To determine that the cell isolation process had not led to t-tubule loss in rat atrial cells, we also performed confocal imaging of intact hearts stained with FM4-64; both right and left ventricular myocytes had a well-developed t-tubule network, whereas t-tubules were again essentially absent in both the left and right atria with the occasional short potential membrane invagination present in few cells (Online Figure III). However, in the sheep atrium, a well-developed t-tubule network was present (Figure 1Ac). As a major role of the surface membrane and t-tubules is to facilitate Ca$^{2+}$ flux in and out of the cell, we next assessed interchamber and species differences in t-tubule occurrence on the distance any point is inside the cell to the surface membrane or t-tubule membrane. This was assessed using 2 approaches; first, as described previously, by calculating distance any point is inside the cell to the surface membrane or t-tubule membrane. This was assessed using 2 approaches; first, as described previously, by calculating distance any voxel is within the cell (in either the vertical or horizontal planes) from a membrane (surface sarcolemma or t-tubule) and calculating the distance which 50% of voxels are from a membrane (referred to as the half-distance, Figure 1Ba–1Bc; Online Figure II). The second approach provides a measure of t-tubule density independent of the effects of cell width on half-distance measurements. Here, we determined the fraction of all voxels within the central 2-µm-thick section of the cell (excluding surface sarcolemma) occupied by t-tubules identified using either di-4-ANEPPS or Alexa Fluor 488–conjugated WGA staining (referred to as the t-tubule fractional area; Figure 1Ca–1Cc). It is clear that, in all species, there are more t-tubules in the ventricular samples than the corresponding atrial samples (Figure 1B and 1C; $P<0.01$). Moreover, in agreement with previous studies, the fractional area occupied by t-tubules in the atrium was greatest in the sheep (Figure 1C).

We next sought to determine whether the interchamber differences in t-tubule density were associated with changes in AmpII protein levels. Representative immunobots from rat, ferret, and sheep samples are shown in Figure 2A. Despite the absence or limited presence of t-tubules in the atria of rat and ferret, AmpII protein is detectable, although at lower levels compared with the respective ventricle (lower by 61±4.0%; $P<0.001$ and 38.2±8.1%; $P<0.05$ respectively). In the sheep myocardium, as is observed in skeletal muscle and mouse ventricle, 2 major isoforms were detected and densitometric analysis of each band alone or both combined yielded the same qualitative result (not shown); we therefore quantified both bands. In the sheep atrium, where the t-tubule network is relatively well developed, AmpII levels were still lower than in the corresponding ventricle. However, the extent of the decrease (26.7±6.7%; $P<0.05$) is less than that in rat and ferret where t-tubules are absent.

The membrane bridging protein JPH2 has also been implicated in determining t-tubule orientation and formation. We therefore examined whether the abundance of JPH2 protein differs between cardiac chambers in line with the changes in t-tubule density and AmpII protein expression noted above. These data are summarized in Figure 2B and show that, irrespective of the presence or absence of t-tubules, there are no interchamber differences in JPH2 in the ventricle, 2 major isoforms were detected and densitometric analysis of each band alone or both combined yielded the same qualitative result (not shown); we therefore quantified both bands. In the sheep atrium, where the t-tubule network is relatively well developed, AmpII levels were still lower than in the corresponding ventricle. However, the extent of the decrease (26.7±6.7%; $P<0.05$) is less than that in rat and ferret where t-tubules are absent.

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**Figure 1.** Quantification of species and interchamber differences in transverse tubule (t-tubule) density. **A**, Representative examples showing membrane staining (upper) and distance maps (lower) in ventricular and atrial myocytes from rat (a), ferret (b), and sheep (c). Cells have been stained with either di-4-ANEPPS (a and c) or WGA (b). Scale bars=10 µm. **B**, Mean data summarizing the distance 50% of voxels (half-distances) are from the nearest cell membrane (t-tubule or surface sarcolemma) in ventricular and atrial cells from (a–c) rat, ferret, and sheep. **C**, Mean data summarizing the fraction of intracellular pixels occupied by t-tubules in ventricular and atrial cells from (a–c) rat, ferret, and sheep. **$P<0.01$; ***$P<0.001$. Rat ventricle, 20 cells/5 hearts; rat atria, 15 cells/3 hearts; ferret ventricle, 8 cells/4 hearts; ferret atria, 8 cells/4 hearts; sheep ventricle, 20 cells/5 hearts; and sheep atria, 21 cells/5 hearts.
Conversely, in the sheep atrium where t-tubules are well developed, JPH2 levels are lower than in the ventricle (by 32.1±6.5%; P<0.001).

**Reduced AmpII or BIN-1 Expression in HF and T-Tubule Loss**

HF is known to lead to loss of t-tubules in the ventricle and atrium and therefore we next determined whether changes in AmpII or JPH2 protein levels occur in parallel to t-tubule loss. First, we examined t-tubule density in ventricular and atrial cells in an ovine tachypacing model of HF. Clinical signs of HF including breathlessness and lethargy were present after 50.5±4.3 days of right ventricular tachypacing. As reported previously, left ventricular internal diastolic dimension increased (pre-pacing, 2.41±0.14 cm; HF, 3.87±0.09 cm; P<0.001) and fractional shortening decreased (pre-pacing, 0.68±0.02; HF, 0.27±0.02; P<0.001) with the development of HF (Online Table I). Isolated myocytes were stained with di-4-ANEPPS to visualize the t-tubule network and it is clear that in both ventricular (Figure 3A) and atrial cells (Figure 3B) there is t-tubule loss in HF. We characterized the extent of t-tubule loss by determining the half-distance value and fractional area occupied by t-tubules (Figure 3Aa and 3Bb). The original images and voxel distance maps show that in the ventricle t-tubule loss was evident at the cell end and to varying extents throughout the cell. Conversely, in the atrium there was an almost complete loss of t-tubules in HF and correspondingly larger increase in the half-distance and smaller fractional area occupied by t-tubules compared with the ventricle. The half-distance increased from 0.38±0.01 to 0.45±0.01 µm in the ventricle (P<0.001) and 0.78±0.07 to 1.94±0.12 µm in the atrium (P<0.01) and the fractional area occupied by t-tubules...
density and decreases the fractional area occupied by t-tubules in HF, resulting in an increase in the aortic coarctation

**Figure 4. Decreased transverse tubule (t-tubule) density and amphiphysin II (AmpII) protein levels but unchanged junctophilin-2 (JPH2) protein levels in ventricular cells after aortic coarctation induced heart failure (HF) in the ferret.**

A. Representative images showing membrane staining (a), distance maps (b), half-distance summary data (iii), and t-tubule fractional area summary data (iib) from control (left) and HF ferret ventricular myocytes (right). Data from (cells/heart); control, 22/7; HF, 15/5. B. Representative Western blots (upper) and summary data for AmpII (a) and JPH2 (b) in control and HF ventricular samples. *P<0.05; **P<0.01; ***P<0.001. For Western blots data are presented normalized to the internal control (IC) sample. Data from 5 control and 6 HF animals. Scale bars=10 μm.

Decreasing from 0.22±0.01 in control to 0.15±0.01 in HF in the ventricle (Figure 3Abi; P<0.001) and from 0.075±0.01 in control to 0.014±0.003 in HF in the atrium (Figure 3Bii; P<0.05). The reduction/loss of t-tubules in the ventricle and atrium in HF was associated with a reduction in AmpII protein levels in both chambers (Figure 3Ca and 3Cb; ventricle by 24.1±5.7% and atrium by 34.5±6.9%; both P<0.05). However, despite the loss of t-tubules in HF, there was no change in JPH2 protein levels in either the ventricle or the atrium (Figure 3D).

To establish that the changes noted above were not restricted to the tachypacing model used in the sheep, we also examined the t-tubule network, AmpII and JPH2 protein levels in a thoracic aortic coarctation/pressure overload model of HF in the ferret.**40,41 Clinical signs of HF took 39±2 days to develop after aortic coarctation and resulted in an increase in left ventricular end-diastolic dimensions from 1.99±0.03 to 2.25±0.06 cm and decrease in ejection fraction from 0.41±0.04 to 0.11±0.02 (both P<0.005; Online Table II). In the HF ventricle, t-tubule loss was evident (Figure 4A) resulting in an increase in the voxel half-distance from 0.294±0.008 μm in sham-operated hearts to 0.353±0.007 μm in HF (P<0.01). The increase in half-distance was accompanied by a decrease in the fractional area occupied by t-tubules in the ferret ventricle in HF from 0.236±0.007 in control to 0.186±0.005 in HF (Figure 4Abi; P<0.001). As in the sheep tachypacing model of HF, AmpII protein levels were decreased in the ferret aortic coarctation model (Figure 4Ba, by 61.3±5.6%; P<0.05) but no change in JPH2 protein levels was observed (Figure 4Bb). Figure 5A examines the relationship between t-tubule density and AmpII protein levels across each of the species, cardiac chambers, and disease models studied thus far. Because different loading controls were used in each experiment, we have normalized the AmpII protein levels to those in the appropriate control ventricular sample. A significant correlation exists between t-tubule half-distance (density) and AmpII protein levels (P<0.01) indicating that, in common with Hong et al,27 t-tubule density depends on AmpII protein levels. Importantly, although the relationship between t-tubule density (half-distance) and AmpII protein seems to diverge at low AmpII levels, this significant correlation is maintained if the sheep is examined in isolation or if the rat atria, which lacks t-tubules, are excluded from the analysis (both P<0.05; data not shown).

**AmpII or BIN-1 Gene Silencing Reduces T-Tubule Density and Increases the Heterogeneity of the Systolic Ca2+ Transient**

We next sought, using siRNA-mediated gene silencing, to determine the role AmpII plays in t-tubule maintenance and synchronization of the systolic Ca2+ transient. The immunolocalization in Figure 5B shows that AmpII has a sarcomeric (≈2-μm spacing) distribution in freshly isolated and scrambled siRNA–transfected rat ventricular cells. However, in AmpII–targeted siRNA (target)–transfected cells the distribution of AmpII is markedly altered becoming noticeably punctate and heterogeneous. The altered AmpII immunolocalization after siRNA gene silencing is associated with disorganized t-tubule staining (Figure 5Bb). Consistent with previous studies showing some degree of t-tubule loss in cultured ventricular cells,**45,46 there was a slight increase in half-distance and decrease in fractional area occupied by t-tubules in scrambled siRNA–treated cells compared with freshly isolated cells (Figure 5D; half-distances: freshly isolated, 0.32±0.02 μm; scrambled siRNA treated, 0.38±0.01 μm, P<0.05; fractional areas: freshly isolated, 0.21±0.01; scrambled siRNA, 0.17±0.01, P<0.05). However, siRNA gene silencing had a more pronounced effect on half-distance and fractional occupation with the siRNA–mediated 12±3.2% decrease in AmpII protein abundance (Figure 5C; P<0.001) resulting in an increase in the voxel half-distance from 0.37±0.01 to 0.57±0.01 μm (Figure 5D; P<0.001) and a decrease in the fractional area occupied by t-tubules from 0.17±0.01; target, 0.08±0.01 (Figure 5D; P<0.001). Importantly, siRNA-mediated gene silencing did not alter JPH2 protein abundance (Online Figure IVA).

Given the potential role of AmpII in trafficking the LTCC to the t-tubule,**26 we also examined the cellular distribution of the LTCC, AmpII, and t-tubules in freshly isolated, scrambled siRNA and AmpII–targeted siRNA–transfected cells (Online Figure V). In freshly isolated cells (Online Figure VA), AmpII and the LTCC have regular striated intracellular distribution. The LTCC is also present to some extent on the surface sarcolemma; nevertheless, there is strong colocalization of the LTCC and AmpII. In scrambled siRNA–treated cells t-tubules were visualized by WGA staining and are clearly well maintained as is the cellular distribution of, and colocalization with, the LTCC (Online Figure VB). After AmpII siRNA–mediated gene silencing there
Figure 5. Transverse tubule (t-tubule) density correlates with amphiphysin II (AmpII) protein levels across tissues, differing disease states and after small interfering RNA (siRNA)–mediated gene silencing. A, Dependence of t-tubule half-distances on AmpII protein levels and the voxel half-distance as a measure of t-tubule density. These data are summarized in Figure 5E and show an inverse correlation between voxel half-distance and AmpII protein levels (P<0.01). Thus, the density of t-tubules depends on the level of AmpII within cardiac myocytes.

Finally, we sought to determine the effect that AmpII gene silencing–mediated t-tubule loss had on the systolic Ca²⁺ transient. Cells were field stimulated and changes in [Ca²+]i were measured using x-t scanning confocal microscopy (Figure 6A). In scrambled siRNA–transfected cells the systolic Ca²⁺ transient rose synchronously along the length of the cell, whereas in the target siRNA–treated cell there are areas where the rise of [Ca²+]i was delayed. On average the time for the systolic Ca²⁺ transient to reach 50% of its peak at each point along the linescan image (F50) increased from 17.3±0.5 ms in scrambled siRNA–transfected cells to 20.2±0.8 ms in target siRNA–treated cells (Figure 6B; P<0.01). To assess the degree of spatial heterogeneity of the systolic rise of [Ca²⁺], the dyssynchrony index was calculated from the SD of the F50 times along each linescan image. The dyssynchrony of the systolic Ca²⁺ transient increased by 163±33% (Figure 6C; scrambled, 13.9±1.3 ms; target, 36.9±3.2 ms; P<0.001). In addition to the delayed and dyssynchronous rise of [Ca²⁺], the amplitude of the systolic Ca²⁺ transient was also reduced in the target siRNA–treated cells, in this instance by 20.6±4.1% (Figure 6D; P<0.001).

JPH2 Gene Silencing Does Not Affect T-Tubule Density but Does Alter T-Tubule Orientation

The previous experiments point to a key role for AmpII in regulating t-tubule maintenance in cardiac muscle. However, several additional proteins have also been implicated in controlling t-tubule maintenance/formation. We investigated a potential role for the membrane bridging protein JPH2, which is thought to be responsible for tethering the SR to the t-tubule and maintaining t-tubule orientation. First, the cellular localization of JPH2 was examined (Figure 7Aa). In both freshly dissociated and scrambled siRNA–transfected cells JPH2 has a primarily sarcomeric distribution with some surface membrane staining. In JPH2 siRNA–transfected cells there is a 26±5.4% decrease in JPH2 protein levels (Figure 7B; P<0.01) with no changes in AmpII protein abundance detected (Online Figure IVB). Notably however, in JPH2 siRNA–targeted cells the cellular distribution of JPH2 appears more diffuse throughout the cell. The altered JPH2 cellular distribution is reflected in a less organized t-tubule network (Online Figure VC).
Figure 6. Amphiphysin II (AmpII) gene silencing–mediated changes to the systolic Ca²⁺ transient. A, Confocal xt line-scans (left) and line-by-line systolic Ca²⁺ rise times (right) from a representative scrambled (upper) and AmpII siRNA–targeted (lower) rat ventricular cell. B, Mean data showing delayed rise time (Δt) of the systolic Ca²⁺ transient in AmpII–silenced rat ventricular cells. C, Mean data showing increased dyssynchrony index (SD of rise times) in AmpII–silenced rat ventricular cells. D, Mean data showing reduced Ca²⁺ transient amplitude after AmpII gene silencing in rat ventricular cells. **P<0.01; ***P<0.001. Data from (cells/experiments); scrambled, 80/12; target, 94/12.

Discussion

Five main findings are presented in this article: (1) within a given species there are interchamber differences in t-tubule density that are paralleled by differences in AmpII but not JPH2 protein levels; (2) t-tubule density and AmpII protein levels, but not JPH2 protein levels, are decreased in 2 distinct models of HF; (3) AmpII gene silencing in adult rat ventricular cells decreased t-tubule density, AmpII protein levels, Ca²⁺ transient amplitude and increased the dyssynchrony of the systolic Ca²⁺ transient, (4) JPH2 gene silencing did not reduce the overall density of t-tubules in rat ventricular cells but did alter t-tubule orientation, and (5) JPH2 has sarcomeric intracellular distribution colocalizing with RyRs in cells lacking t-tubules. Taken together these findings indicate that AmpII has a major role in t-tubule maintenance in cardiac muscle. Conversely, however, JPH2 seems to be more important for controlling t-tubule orientation and localization of nonjunctional RyRs rather than controlling t-tubule density in cardiac muscle.

Differences in T-Tubule Density, AmpII or BIN-1, and JPH2 Protein Levels Between Atrial and Ventricular Cardiac Myocytes

In accordance with many,¹⁴ but not all studies,¹⁵,²² we found that rat atrial myocytes lack a discernible t-tubule network. We also found that the ferret atrium like that of the cat¹⁰ and rabbit¹³ had only a sparse t-tubule network present in some cells. However, as described previously,¹⁸–²⁰ the sheep atrium has a well-developed t-tubule network. Functionally, where the t-tubule network is absent in atrial myocytes the systolic Ca²⁺ transient rises initially at the cell periphery and then propagates as a wave of Ca²⁺–induced Ca²⁺ release to the cell center.¹⁴⁹ However, the relatively well-developed t-tubule network in the
ner t-tubule membrane. Interestingly, in agreement with several previous studies,\textsuperscript{2,5} we do not note inner t-tubule membrane folding in the rat or sheep ventricular myocardium by serial block face scanning electron microscopy\textsuperscript{9} and the $\approx 250 \text{ nm}$ resolving capability of confocal microscopy is insufficient to visualize such structures or subtle changes in t-tubule lumen diameter. As such the inner t-tubule membranes noted by Hong et al\textsuperscript{27} may be features specific to the mouse or reflect the role of species differences in BIN-1 (AmpII) isoform expression.

We find that AmpII protein levels are lower in the atrium compared with the ventricle, which therefore parallels the observed differences in t-tubule density between the atrium and ventricle. Conversely, in the rat and ferret atria where the t-tubule network is either absent or sparse, we do not observe a corresponding decrease in JPH2 protein levels compared with that seen in the ventricle. Paradoxically however in the sheep atrium, where t-tubules are prevalent, JPH2 protein levels are less than that in the corresponding ventricle.

An unresolved question arising from this work is what is the basis of the constancy of JPH2 expression in the rat and ferret atria and ventricle despite the marked differences in t-tubule density? Although we do not have a definitive answer, in the rat atrium where t-tubules are virtually absent, RyRs and LTCCs form dyads at the cell surface.\textsuperscript{54} In addition, at least in some studies, the L-type Ca$^{2+}$ current density in the rat atrium and ventricle is the same\textsuperscript{37,55} suggesting that the surface density of LTCCs and thus dyads may be greater in the atrium than the ventricle. Furthermore, RyRs are distributed throughout the cell as part of the nonjunctional or corbular SR.\textsuperscript{17,56} Thus, JPH2 may still ensure dyad alignment and non-junctional SR alignment in these cells. In support of this we find that RyR and JPH2 have a regular sarcomeric intracellular distribution and colocalize in sheep HF atrial myocytes and rat atrial myocytes where t-tubules are absent.

A subsidiary question is why, despite the marked difference in t-tubule density between the atria and ventricles in the rat and ferret, is there only slightly less AmpII protein in the atrium compared with the ventricle? We propose that the explanation for this observation is possibly because of the plurality of roles for AmpII. In a recent study by Hong et al\textsuperscript{27} and in Drosophila indirect flight muscle,\textsuperscript{29} loss of AmpII is associated with t-tubule loss. Conversely, AmpII also seems to be required for trafficking of the LTCC to the cell membrane in cardiac myocytes.\textsuperscript{26} JPH2, however, tethers the SR/RyR to the sarcolemma/Z-line maintaining the geometry of the dyad.\textsuperscript{44,51} Deletion of either AmpII and JPH2 results in perinatal lethality with evidence of cardiac failure and structural disorganization.\textsuperscript{28,44,44} It is also noteworthy that the recently described murine cardiac conditional BIN-1 (AmpII) knockout heterozygote heart retains a regular, albeit less intensely stained, t-tubule network.\textsuperscript{27} However in the same study, short hairpin RNA gene silencing of AmpII caused t-tubule loss as was observed herein and moreover, adenoviral-mediated expression of the 13+17 splice variant of BIN-1 increased t-tubule intensity in cultured heterozygote cells. In addition, in the BIN-1 knockout heart the main secondary effect of BIN-1, after maintaining t-tubule density, seems to be the generation of a tightly folded inner t-tubule membrane. Interestingly, in agreement with several other studies,\textsuperscript{2,53} we do not note inner t-tubule membrane folding in the rat or sheep ventricular myocardium by serial block face scanning electron microscopy\textsuperscript{9} and the $\approx 250 \text{ nm}$ resolving capability of confocal microscopy is insufficient to visualize such structures or subtle changes in t-tubule lumen diameter. As such the inner t-tubule membranes noted by Hong et al\textsuperscript{27} may be features specific to the mouse or reflect the role of species differences in BIN-1 (AmpII) isoform expression.

Remodeling of T-Tubules in HF

Further evidence for the importance of AmpII in t-tubule maintenance is provided by the observation that in the 2 different models of HF used in the present study there is a reduction in t-tubule density paralleled by a decrease in AmpII but not JPH2 levels. Several previous studies have reported t-tubule loss and disorganization in cardiac disease...
states,7-9,18,19,48 whereas some, in line with the present study, have reported reductions in AmpII11,25,26 and others have also reported reductions in JPH2 in HF.11,42,48 It is noteworthy however that, in response to SR Ca2+-ATPase (SERCA) gene delivery11 or mechanical unloading9 as treatments for HF, t-tubule density7–9,18,19,48 and AmpII (BIN-1) protein levels11 increased toward control levels but those for JPH2 remained reduced. Similarly, in a pulmonary hypertension model of right ventricular failure sildenafil treatment commenced when ventricular dysfunction was evident leads to an increase in t-tubule density but not of JPH2 protein levels.57 Conversely, β-blocker therapy commenced after myocardial infarction was associated with an increase in t-tubule density and JPH2 protein relative to failing tissues.58 However, these discrepant findings most likely reflect differences in study design and commencement of β-blocker therapy before ventricular dysfunction was evident and hence attenuation of the progression of HF rather than recovery from HF; a situation distinct from those noted above for the effects of mechanical unloading and SERCA gene therapy.9,11

Gene Silencing Approaches Highlight an Important Role for AmpII or BIN-1 in T-Tubule Maintenance in Cardiac Muscle

The interchamber and HF differences in t-tubule density and AmpII expression described above, while indicating an important role for AmpII in t-tubule maintenance in cardiac muscle, remain as only associative observations. We therefore sought to define more precisely if reductions in AmpII expression directly influence t-tubule density in cardiac muscle and adopted a siRNA transient transfection approach in adult rat ventricular myocytes. Here, we observed a direct relationship between AmpII protein and t-tubule density even after a relatively short period of ≈24-hour gene silencing. A similar linear dependence of t-tubule intensity on AmpII protein in cardiac myocytes has also been reported recently using lentiviral-mediated short hairpin RNA gene silencing during an extended 4-day culture period27; however, whether the extended culture period also influences these latter observations is unclear given the propensity of adult cardiac cells to dedifferentiate relatively rapidly when maintained in culture conditions.46 The reported-half life of AmpII is ≈2 hours50 and therefore amenable to transient transfection techniques and short-term culture of adult ventricular myocytes was therefore used in the present study. Given the low transfection efficiency of adult cardiac myocytes when using nonviral approaches,46 the reduction in AmpII protein levels after siRNA treatment (≈12%) is likely an underestimate of the reduction in AmpII in individual cells that have been successfully transfected; although cells were randomly studied, identifying which cells had been successfully transfected using fluorescently labeled siRNA was not successful in the present study.

Although AmpII gene silencing leads to a loss of t-tubules and therefore an increase in the distance any point within the cell is from a t-tubule or surface membrane, we found that both the half-distance and the fraction of the cell volume occupied by t-tubules were unaltered by siRNA-mediated JPH2 gene silencing. However, JPH2 gene silencing did result in an alteration to the spatial arrangement of the t-tubules from a predominantly transverse (perpendicular to the long axis of the cell) orientation to one where axially arranged t-tubules were frequently observed. A similar t-tubule reorientation as a consequence of JPH2 gene silencing has been noted previously.42 More recently, it has also been suggested that, during postnatal development, JPH2 may have an important role in determining the formation of transversely oriented t-tubules rather than axially arranged t-tubules as the transversely oriented t-tubules were found to persist after JPH2 gene silencing.61 Similarly, in those models of HF where JPH2 levels are decreased, an increase in the proportion of axially arranged t-tubule elements has been noted.48,62 Although in the present study JPH2 was not reduced in either the sheep tachypacing or ferret pressure overload models of HF, there was a change in t-tubule orientation in the sheep tachypacing model. This implies that in addition to JPH2, AmpII, potentially even specific AmpII isoforms or other factors may also be responsible for maintaining t-tubule orientation. However, it seems that our findings are most consistent with AmpII being required for t-tubule maintenance and that JPH2 has a role in ensuring the correct spatial alignment of t-tubules and RyRs in the heart.

Consequences of T-Tubule Loss on the Systolic Ca2+ Transient

In the present study, we show that AmpII gene silencing—mediated depletion of t-tubules leads to a reduced Ca2+ transient amplitude and dysynchrony of the systolic Ca2+ transient. Previous studies have also shown that t-tubule disorientation after JPH2 gene silencing in various cell types also increases the heterogeneity of the systolic Ca2+ transient.44,61,63 Our findings are also in line with earlier studies showing that the systolic Ca2+ transient amplitude is reduced and becomes dysynchronous in HF.7,9,11 In HF, there is also a reduction in the t-tubule density, although the loss of t-tubules has not been previously shown to be causative of the dysynchronous Ca2+ transient. However, acute formamide–induced detubulation of cardiac myocytes and extended culture of adult cardiac myocytes also lead to a reduction in L-type Ca2+ current and reduced synchronicity of the systolic Ca2+ transient, suggesting a causal link between t-tubule disruption and Ca2+ transient heterogeneity.1,46,64,65

In summary, we show that the Bin/Amphiphysin/Rvs domain protein AmpII (BIN-1) is intrinsically involved in the maintenance of cardiac t-tubules and thus ensuring the synchronicity of the systolic Ca2+ transient. Our data also support a role for JPH2 in maintaining the normal orientation of t-tubules. It is therefore possible that AmpII and JPH2, along with several other proteins implicated in t-tubule formation, for example, titin cap protein (telethonin) and phosphoinositide 3-kinase (PI3K), may form a signaling nexus along the z-line to regulate t-tubule formation, maintenance, and orientation. However, from the present study it is clear that t-tubule maintenance depends on AmpII levels and that changes in t-tubule density correlate strongly with AmpII in both the healthy and diseased heart. Thus, AmpII may be an attractive target for restoring t-tubules and thus systolic Ca2+ and contractility in HF.

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39. Horn MA, Graham HK, Richards MA, Clarke JD, Greensmith DJ, Briston SJ, Hall MC, Dibb KM, Trafford AW. Age-related divergent remodeling of


Dependence of Cardiac Transverse Tubules on the BAR Domain Protein Amphiphysin II (BIN-1)
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Dependence of cardiac transverse (t) tubules on the BAR domain protein Amphiphysin II (BIN-1)


The University of Manchester, Institute of Cardiovascular Science, Manchester UK.

Methods

All procedures involving the use of animals were performed in accordance with The United Kingdom, Animals (Scientific Procedures) Act, 1986 and European Union Directive 2010/63/EU. Local Institutional approval was obtained from The University of Manchester Animal Welfare and Ethical Review Board. The detailed reporting of in vivo experiments in the supplementary information is in line with the ARRIVE guidelines 1.

Animals (rats, ferrets, sheep) were group (4 – 6 animals per cage) housed (rats in individually ventilated cages) on a 12:12 light:dark cycle, 19 - 21 °C, had ad libitum access to drinking water and were fed standard laboratory rations (rats, ferrets) or hay and ruminant concentrate (sheep). Environmental enrichment e.g. tunnels were provided as appropriate to the species being studied.

Isolation and t-tubule imaging of rat cardiac myocytes – Single atrial and ventricular myocytes were isolated from 20 male Wistar rats (~ 250 g. Charles River, UK) killed by stunning and cervical dislocation using a collagenase digestion technique based on previously described methods 2. Following isolation cells were maintained in a 5% CO2 - 95% air atmosphere at 37°C in Myocyte Growth Medium (Promocell) supplemented with (in mmol/L unless stated): insulin, 0.0001; blebbistatin, 0.1 mg/ml; creatinine, 2; taurine, 5; Na-pyruvate, 2; L-carnitine, 2. Cells were stained with di-4-ANEPPS (4 μmol/L for 10 mins) and t-tubules visualised using a Leica SP2 confocal microscope 3,4. Images were deconvolved using Huygens Essential (SVI, Netherlands) and the microscopes point spread function and t-tubule density assessed as the distance at which 50 % of the voxels within the cell are from a cell membrane (t-tubule or surface sarcolemma) 3,4. To quantify t-tubule orientation images were thresholded, binary converted, skeletonised and t-tubule orientation probability histograms obtained using the FIJI ‘directionality’ algorithm 5.

Whole heart t-tubule imaging – following euthanasia hearts from Wistar rats were perfused (at 4 °C) via the aorta with a Ca2+ free Hanks buffered salt solution (HBSS; Sigma, UK) to which 1 mmol/L EDTA was added. The styryl dye FM4-64 (2.5 μmol/L;
Invitrogen UK) \(^6\) was then perfused, with recirculation, for 20 mins (4 °C). The heart was then perfused with Ca\(^{2+}\) free HBSS / EDTA solution for 10 mins (4 °C). The heart was then fixed on the stage of an inverted Zeiss 7Live confocal microscope and the epicardial surfaces of the left and right atria and ventricles imaged (~ 15 fields of view each chamber). Fluorescence was excited at 488 nm (Airy 1 pinhole) and emitted light collected > 600 nm. The atria were then separated from the ventricles and the endocardial atrial surfaces imaged as above.

**Small interfering RNA transfection of isolated rat ventricular myocytes** – Adult rat ventricular myocytes were seeded at approximately 1 x 10\(^5\) / ml per well on laminin (20 ug/ml, Life Technologies) coated 6 well culture plates. Myocytes were maintained in a 5 \% CO\(_2\) - 95 \% air atmosphere at 37 °C for 1 hour. Cultured adult rat ventricular myocytes were transfected with siRNA constructs as per manufacturers instructions (Santa Cruz Biotechnology) either against Ampl (Sigma Mission siRNA; SAS1_Rn01_00102283/ 00102284 / 00266922) or JPH2 (Sigma Mission siRNA SASI_Rn01_00049616) at a concentration of 10 μmol/L. After 7 hours transfection, Myocyte Growth Medium (Promocell) containing 10 % Fetal Bovine Serum (FBS) (Gibco) and 2 % penicillin streptomycin (PenStrep) (10,000 units penicillin & 10mg/ml streptomycin, Invitrogen) was added to the cells. Myocytes were then maintained in a 5 \% CO\(_2\) - 95 \% air atmosphere at 37 °C overnight. Myocytes cultured under identical conditions and transfected with scrambled siRNA (sc-37007, Santa Cruz Biotechnology, 10 μmol/L) served as controls. After 24 hours transfection proteins were extracted and used for Western blotting and t-tubule imaging was carried out as described above.

**Quantification of Ca\(^{2+}\) transient dyssynchrony** – Cells were loaded with the Ca\(^{2+}\) sensitive fluorescent indicator Fluo-3 AM (5 μmol/L, 30 mins) and allowed to de-esterify for at least 30 mins before being confocally imaged at 23 °C. Cells were electrically stimulated via a pair of platinum wires in the perfusion chamber. Confocal line (xt) scans were obtained along the long-axis of the cell. Images were analysed using IDL software (Exelis Visual Information Solutions, UK) and pseudo-ratioed relative to the resting fluorescence before the cell was electrically stimulated. The time to reach 50 % of the maximum pseudo-ratio (F\(_{50}\)) at each point along the scan line was then calculated and the standard deviation of all the F\(_{50}\) values used as the dyssynchrony index of the systolic Ca\(^{2+}\) transient \(^7,8\).

**Tachypacing induced heart failure in the sheep** – A total of 39 adult female Welsh sheep were included in the study and animals were randomly assigned to control (non-instrumented) or heart failure groups. Heart failure was induced in 17 ewes (29.9 ± 1.5 kg) by right ventricular tachypacing as described previously \(^4,9,10\). Anaesthesia was induced and subsequently maintained by isoflurane inhalation (1 – 4 %), animals were intubated and ventilated at 15 breaths per minute and analgesia (meloxicam 0.5 mg.kg\(^{-1}\)) and antibiosis (enrofloxacin 5 mg.kg\(^{-1}\)) provided. A single IS-1 bipolar pacing lead was fixed transvenously at the right ventricular apex and connected to a pacemaker (Kappa, Medtronic) buried in a subcutaneous pocket. Animals were allowed to recover from surgery for at least 1 week before tachypacing (3.5 Hz) was commenced. Animal welfare post operatively and the onset of clinical signs of heart failure were monitored at least once daily by competent trained personnel. On presentation of clinical signs of heart failure (lethargy, dyspnoea, cachexia) animals were killed (pentobarbitone 200 mg.kg\(^{-1}\) intravenously with heparin 10,000 units intravenously to prevent coronary coagulation) and single ventricular myocytes isolated using a collagenase and protease digestion technique as previously described \(^10\). T-tubule density was assessed using di-4-ANEPPS
as described above. In vivo cardiac function was assessed in conscious, unsedated gently restrained animals using a parasternal echocardiography (Sonosite Micromaxx, BCF Technology UK) and images analysed offline in ImageJ (NIH, USA) \(^9,10\).

**Ferret atrial and ventricular t-tubule assessment** – Following pentobarbitone (200mg.kg\(^{-1}\) intraperitoneal) euthanasia hearts were removed and sections of left atrial and left ventricular tissue fixed in 4 % paraformaldehyde solution for 24 hours before being processed through graded alcohols and embedded in paraffin wax blocks \(^11\). Sections (5 \(\mu\)m) were prepared, de-waxed, subject to heat-mediated antigen retrieval (sodium citrate buffer, 95 °C, 15 mins and allowed to cool for 20 mins) and stained with Alexa Fluor 488 conjugated wheat germ agglutinin (WGA, 1:50 dilution (ventricle) or 1:100 dilution (atria), 1 hour). Sections were then confocally imaged and t-tubule density calculated using the same approach used for di-4-ANEPPS stained cells as described previously \(^3\).

**Aortic coarctation model of heart failure in the ferret** – Heart failure was induced in 6 adult male ferrets (1235 ± 111 g, B & K Universal UK) by ascending aortic coarctation as previously described \(^11,12\). Briefly, under isoflurane (1 – 3 % v/v) anaesthesia a left parasternal thoracotomy was performed, the ascending aorta isolated and enclosed by an inflatable vascular occluder (Harvard Apparatus). The thorax was then surgically closed and the occluder connected to an osmotic pump (Charles River) located subcutaneously on the dorsum. Post-operative analgesia was provided with meloxicam (0.5 mg/kg) and antibiotics with enrofloxacin (5 mg/kg). Sham operated animals (\(n=7\)) served as controls. Animals were assigned to heart failure or sham surgical procedures randomly. Sham animals underwent left lateral thoracotomies and aortic root localisation as for heart failure animals but not subjected to aortic banding. Animal welfare post operatively and the onset of clinical signs of heart failure were monitored at least once daily by competent personnel and when present (lethargy, cachexia, ascites, dyspnoea) animals were humanely killed (pentobarbitone, 200 mg.kg\(^{-1}\) intraperitoneally). Sham operated animals were humanely killed at time-matched points. In vivo cardiac function was assessed in isoflurane (1 – 3 % v/v in oxygen) anaesthetised animals using a Siemens Sequoia 512 and analysed offline using ImageJ (NIH, USA)

**Protein isolation, Amphiphysin II and Junctophilin 2 quantification** – Ventricular and atrial tissue samples and isolated cells were collected and homogenised in RIPA buffer containing protease and phosphatase inhibitors (0.1 mg/mL phenylmethanesulphonylfluoride, 100 mmol/L sodium orthovanadate, 1mg/mL aprotonin, 1mg/mL leupeptin). Samples were prepared for separation using Novex 4-12% Bis-Tris gels (NuPAGE, Life Technologies) as described previously \(^10\). Following electrophoresis samples were transferred to nitrocellulose membranes (GE Healthcare, UK). Membranes were then blocked with Superblock or SEABLOCK® (Thermo Scientific, UK) and probed with primary antibodies that detect AmplI (1:5000, Santa Cruz Biotechnology) or JPH2 (1:1000, Santa Cruz Biotechnology, USA). HRP conjugated secondary antibodies anti-mouse IgG (1:40,000) and anti-goat IgG (1:5000) were used with chemiluminescence substrate (GE Healthcare) to detect proteins of interest and captured digitally (Genesnap, Syngene UK). Three technical replicates were performed for each blot and data averaged. For Western blots from rat and ferret tissue and cells, membranes were stripped (Western blot restore, Thermo Scientific UK) and re-probed with with primary antibodies that detect house-keeping proteins GAPDH or \(\beta\)-actin (both 1:5000, Santa Cruz Biotechnology, USA). Protein was quantified relative to house-
keeping protein. For Western blots of heart failure tissue however, classic ‘housekeeping’ proteins β-actin and GAPDH have been shown to change (Online Figure I). Therefore an internal standard (IC), obtained from a single control animal of the same species, was loaded on all blots. This internal protein standard was used to normalize protein levels on each gel as described previously 9,10. Importantly, normalisation of protein levels to this internal control protein yielded results indistinguishable from those when protein levels were normalised to protein loading assessed from Ponceau S stained membranes (Online Figure I).

**Immunocytochemistry** – Myocytes were fixed and permeabilized in ice-cold acetone for 7 minutes, blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.25% triton-X 100 (PBS-T) and probed with primary antibody that detect AmpII (1:100, Santa Cruz Biotechnology), JPH2 (1:100, Santa Cruz Biotechnology), L-type Ca$^{2+}$ channel (1:50 Novus Biologicals) and RyR (1:1000, Abcam). Secondary antibodies (Goat Anti-Mouse, A11001; Donkey Anti-Goat, A11055, Molecular Probes) 20ug/ml conjugated to Alexa Fluor® 488 or Alexa Fluor® 647 were then applied to cells. Following incubation with secondary antibody, Vectashield with 1.5ug/ml 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs), was used to mount coverslips. Slides were then confocally imaged and cellular protein distribution imaged as described previously 3.

Images were acquired on a Leica SP2 confocal microscope (λex 488 nm or 633 nm; λem 500 – 600 nm and > 640 nm for Alexa Fluor 488 and 647 respectively). Using Huygens Professional (Scientific Volume Imaging, Netherlands) and the microscopes point spread function (PSF, 100 nm diameter TetraSpeck beads, Invitrogen UK 2,4), images were thresholded and deconvolved before colocalisation analysis was performed (Huygens Professional). Colocalisation of the RyR, JPH2, t-tubules (WGA-Alexa Fluor® 647 stained), LTCC and AmpII as appropriate were assessed using Manders coefficients 13 (Huygens Professional).

**Statistics** – Prior to the commencement of the study Power Analysis (Sigmaplot Software, Systat UK) was performed to estimate required sample sizes assuming a desired power (β) of 0.8 and cut off (α) for significance of 0.05 based on previously published differences and data standard deviations in the models used in this study 2,4,9,10,12,14. Once an animal was enrolled in the study and data acquired it was not subsequently excluded from analysis. Normality of data distribution was assessed using the Kolmogorov-Smirnov statistic and normally distributed data are presented as mean ± standard error of the mean (SEM) whilst the median (inter-quartile range) are used for non-normally distributed data. In each case data are presented as n cells from N experimental subjects (e.g. n/N atria; n/N ventricle). Continuous scale data was log$_{10}$ transformed 15 and differences between groups determined using linear mixed modeling (IBM SPSS Statistics v20) to account for multiple observations (e.g. cells) from each experimental subject (i.e. animal). Where treatments or observations were obtained from the same experimental animal (e.g. echocardiographic data pre-pacing induced heart failure and at end-stage heart failure) differences were assessed using a paired Students t-test and considered significant when $P < 0.05$. Pearsons correlation coefficient was used to determine if significant relationships existed between bivariate data and considered significant when $P < 0.05$. For immunohistological colocalisation analysis Manders coefficients were calculated using Huygens Professional (Scientific Volume Imaging, Netherlands).
Results

*Loading controls: Inter-chamber and heart failure associated changes in house-keeping proteins in the heart*

A key requirement when assessing changes in protein levels between tissues and in disease states is adequate normalisation to a control protein. In many instances β-actin or GAPDH are used to serve this purpose and membranes are stripped and re-probed for these ‘control’ proteins following initial immuno-blotting against the primary protein of interest. However, as is illustrated in Online Figure I.A-D both β-actin and GAPDH show differences in protein levels in the sheep ventricle and atria and as a result of heart failure. As an alternative to normalisation to these ‘control’ proteins we have therefore used an internal standard (IC) common to each blot and normalised protein levels to this standard. We have, however, also validated this data by normalising the levels of our protein of interest to the total protein transferred to the nitrocellulose membrane assessed by Ponceau S staining (Online Figure I.E-G). Importantly no qualitative differences in our results were found when employing the internal standard or total protein (Ponceau S) approaches.

![Graphs showing protein levels](image.png)

**Online Figure I. House-keeping and loading controls for immunoblotting.**

A-D. Representative immuno-blots (upper panels) for β-actin (A, C) and GAPDH (B, D) and summary data (lower panels) detailing inter-chamber (A, B) and heart failure associated (C, D) changes in protein levels of ‘control’ proteins. In each case protein...
levels have been normalized to an internal standard (IC) sample common to each blot and technical replicate. The internal control sample was obtained from a ‘control’ ventricular sample from the same species as the atrial or ventricular samples being studied. E-G. Representative Ponceau S stained membranes (upper) and resulting immunoblots (middle) and summary data (lower) illustrating equal protein loading between samples. When protein levels were normalized to total protein loaded AmpII (E) and JPH2 (F) protein levels are lower in the sheep atria than in the sheep ventricle. However, in heart failure JPH2 abundance in the sheep ventricle (G) is unaltered. IC, internal standard (a control ventricular sample); MW, molecular weight marker; *, P < 0.05; **, P < 0.01. N = 6 – 7 hearts and 3 technical repeats for each blot.

Quantification of voxel half-distances to surface sarcolemma and t-tubules

We have used two approaches to quantify t-tubule density in cardiac myocytes and tissue sections stained with either di-4-ANEPPS or Alexa Fluor 488 conjugated WGA. Firstly, as previously described \textsuperscript{3,4}, we calculate the half-distance; the distance within which 50% of voxels are from either the surface sarcolemma or t-tubule membrane. Under these circumstances, following image deconvolution and thresholding the distance any voxel is from the nearest membrane (t-tubule of surface sarcolemma) is determined in the x, y and z dimensions. The calculated data using this approach is highlighted in Online Figure II. However, the half-distance calculation suffers from the fact that cell width also influences the calculated half-distance. To overcome this limitation we have also calculated the fractional area of the cell occupied by t-tubules to obtain a cell-width independent assessment of t-tubule density (figure 1C, main manuscript). The fractional area occupied by t-tubules was calculated by digitally removing the surface membrane using the polygon selection and ‘clear outside’ routines in FIJI (Image J). Following thresholding and binary conversion the fraction of white (membrane stain) pixels of the total pixels within the image was calculated.

![Graphs showing the quantification of voxel half-distances to surface sarcolemma and t-tubules](image)

**Online Figure II. Distance to membrane plots in ventricular and atrial myocytes from the rat, ferret and sheep.**

The plots show relationship between the cumulative fraction of pixels (ordinate) and distance (abscissa) from a membrane structure in typical rat (a), ferret (b) and sheep (c)
ventricular (black) and atrial (grey) myocytes. The solid lines represent data for effects of the surface sarcolemma and t-tubules combined whereas the dashed lines show the contribution from the t-tubules alone (obtained by digitally removing the surface sarcolemma 3,4). The half-distance is obtained by determining the distance value corresponding to the 50th centile of voxels 3,4. It is clear that in all ventricular cells the t-tubules is the major determinant of the half-distance whereas in the rat atria the surface sarcolemma (cell-width) determines the half-distance due to the absence of t-tubules.

Echocardiographic indices in tachypacing induced heart failure in the sheep

Trans-thoracic echocardiograms were performed in conscious (unsedated) sheep gently restrained in a sitting position. Long and short axis images were obtained from a parasternal window and at least three cardiac cycles were averaged to obtain each measurement. Each animal (n = 10) served as its own control with baseline measurements obtained prior to the commencement of tachypacing to induce heart failure. Fractional shortening was calculated from long-axis m-mode images whereas the fractional area change was calculated by measuring the endocardial surface in short axis parasternal views.

<table>
<thead>
<tr>
<th></th>
<th>Pre-pacing values</th>
<th>Heart failure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>End Diastolic Internal Dimension (cm)</strong></td>
<td>2.41 ± 0.14</td>
<td>3.87 ± 0.09 §</td>
</tr>
<tr>
<td><strong>End Systolic Internal Dimension (cm)</strong></td>
<td>0.80 ± 0.06</td>
<td>2.84 ± 0.13 §</td>
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<tr>
<td><strong>Fractional area change (short-axis view)</strong></td>
<td>0.68 ± 0.03</td>
<td>0.38 ± 0.03 §</td>
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<tr>
<td><strong>Fractional shortening (long axis view, m-mode imaging)</strong></td>
<td>0.68± 0.02</td>
<td>0.27 ± 0.02 §</td>
</tr>
<tr>
<td><strong>Relative left ventricular free wall thickness</strong></td>
<td>1.02 ± 0.12</td>
<td>0.41 ± 0.03 #</td>
</tr>
</tbody>
</table>

**Supplementary Data, Table I. Cardiac dilatation and reduced contractility following tachypacing induced heart failure in sheep.**

Mean data summarizing echocardiographic parameters. §, P < 0.00005; #, P < 0.005. N = 10 animals.

**Echocardiographic indices in thoracic aortic coarctation induced heart failure in the ferret**
Trans-thoracic echocardiograms were performed in isoflurane (1 – 3 % v/v) anaesthetized ferrets. Long and short axis images were obtained from a para-sternal window and at least three cardiac cycles were averaged to obtain each measurement.

<table>
<thead>
<tr>
<th></th>
<th><strong>Sham Operated</strong> ($N = 6$)</th>
<th><strong>Heart Failure</strong> ($N = 6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>End Diastolic Internal Dimension (cm)</strong></td>
<td>1.08 ± 0.04</td>
<td>1.19 ± 0.05 *</td>
</tr>
<tr>
<td><strong>End Systolic Internal Dimension (cm)</strong></td>
<td>0.62 ± 0.05</td>
<td>1.06 ± 0.04 %</td>
</tr>
<tr>
<td><strong>Free wall thickness (cm)</strong></td>
<td>0.41 ± 0.01</td>
<td>0.47 ± 0.03 * $P = 0.06$</td>
</tr>
<tr>
<td><strong>Fractional shortening (long axis view, m-mode imaging)</strong></td>
<td>0.41 ± 0.04</td>
<td>0.11 ± 0.02 %</td>
</tr>
</tbody>
</table>

**Supplementary Data, Table II. Cardiac dilatation and reduced contractility following aortic banding induced heart failure in ferrets.**

Mean data summarizing echocardiographic parameters. *, $P < 0.05$; %, $P < 0.0001$.

**Whole heart imaging shows absence of t-tubules in the left and right atria of Wistar rats**

Following whole heart perfusion with the membrane dye FM4-64 we confocally imaged the endocardial and epicardial surfaces of the left and right atria and ventricles. A well-defined regular t-tubule pattern was present in both the left and right ventricular images (Online Figure III). However, transverse or longitudinal (transverse axial \(^{16}\)) tubules were lacking in left or right atrial images whether they were imaged from the epicardial or endocardial surfaces (Online Figure III). In atrial samples, the limited intracellular staining noted in some cells was not continuous with the cell surface when image series (z-stacks) were analysed in the planes above and below the central focal plane. Some non-tubular intracellular staining is also present in ventricular cells.
**Online Figure III. Whole heart imaging in the rat.**

FM4-64 stained rat heart imaged confocally from the left and right ventricular and atrial surfaces as indicated. Scale bars 10 μm. Representative of 3 separate experiments (hearts).

**Absence of reciprocal changes in protein levels following siRNA mediated gene silencing of Amphiphysin II or Junctophilin 2**

We have used immunoblotting to determine whether siRNA mediated gene silencing of AmpII or JPH2 result in reciprocal changes in the amount of JPH2 or AmpII protein respectively. Myocytes were plated on to 6 well culture dishes and transfected with either AmpII or JPH2 targeted siRNA as described above and 24 hours post-transfection were prepared for immunoblotting as shown in Online Figure IV. It is clear that neither JPH2 and AmpII protein levels are altered by siRNA mediated gene silencing of AmpII or JPH2 respectively.

**Online Figure IV. No reciprocal changes in Junctophilin 2 or Amphiphysin II protein levels following siRNA mediated gene silencing.**

Representative immunoblots for JPH2 (A, upper), AmpII (B, upper) and β-actin (centre) from myocytes transfected with AmpII siRNA (A), JPH2 siRNA (B). The lower panels summarise mean data showing no change in JPH2 (A) or AmpII (B) protein levels relative to scrambled siRNA transfected cells. In both scrambled and target siRNA treated cells protein levels were normalized to β-actin. N = 3 – 4 experiments.

*L-type Ca²⁺ channels colocalise with AmpII and t-tubules in rat ventricular myocytes*
Previous studies have shown that AmpII facilitates trafficking of the LTCC to the cell surface and is implicated in folding of the t-tubule membrane \textsuperscript{17-19}. Here we sought to determine how acute AmpII siRNA mediated gene silencing impacts on LTCC and t-tubule distribution. In both freshly isolated rat ventricular myocytes and scrambled siRNA transfected cells following 24 hours culture the LTCC has a regular sarcomeric and surface membrane distribution that colocalises with AmpII (Online Figure V.A) and the t-tubule and surface membrane (Online Figure V.B). In AmpII targeted siRNA transfected myocytes the extent of both the t-tubular and LTCC staining is reduced, particularly in the cell centre and there is very little colocalisation (Online Figure V.C).

\textbf{Online Figure V. AmpII, t-tubule and L-type Ca\textsuperscript{2+} channel distribution is altered following AmpII gene silencing.}

\textbf{A.} Representative image showing colocalisation of AmpII (red) and LTCC (green) in freshly isolated rat ventricular myocytes showing extensive colocalisation (yellow; Manders M1 = 0.98; from 2 cells, 1 heart). We have also determined in cells stained
independently (data not shown) that AmpII (20 cells, 3 hearts) and the LTCC (11 cells, 2 hearts) have sarcomeric intracellular distribution. B. Representative images (from 10 cells, 3 hearts) obtained following scrambled siRNA transfection and 24 hours culture t-tubules. Images show that WGA staining (red, t-tubules) and LTCC (green) retain regular sarcomeric distribution and remain colocalised (yellow; Manders M1 (i) 0.92, (ii) 0.89). C. Representative images (from 16 cells, 3 hearts). AmpII siRNA gene silencing mediated t-tubule (WGA staining, red) and LTCC (green) depletion and reduced colocalisation (yellow; Manders M1 (i) 0.23, (ii) 0.29). Following AmpII gene silencing the remaining colocalisation of AmpII and the LTCC is restricted to the cell periphery. Scale bars 10 μm.

Transverse tubule orientation analysis in heart failure

Given the putative role of JPH2 in tethering the SR/RyR to t-tubules and maintenance of t-tubule orientation 6,20-22 we sought to determine if the unchanged JPH2 protein levels noted in the ovine tachypacing and ferret pressure overload models of heart failure was associated with retained t-tubule orientation. Ventricular myocytes were stained with di-4-ANEPPS (sheep) or WGA (ferret) to visualize t-tubules and the resulting confocal images skeletonized and t-tubule orientation analysis performed in ImageJ 5 (Online Figure VI). In the sheep model of tachypacing induced heart failure t-tubule orientation was altered (Online Figure VI.A) with a reduction in the ratio of transverse to longitudinal t-tubules noted (Online Figure VI.B). Conversely, in the ferret pressure overload model of heart failure (Online Figure VI.C) t-tubules retained their predominant, perpendicular to the long axis of the cell, orientation and the transverse to longitudinal ratio was unaltered (Online Figure VI.D).
**Online Figure VI. Transverse tubule orientation is altered in tachypacing induced heart failure but not in pressure overload induced heart failure.**

**A.** Representative control (left) and heart failure (right) di-4-ANEPPS stained (a) and skeletonized (b) images of single sheep ventricular myocytes and t-tubule orientation analysis (c). **B.** Mean data summarizing the change in ratio of transverse to longitudinal t-tubules in tachypacing induced heart failure. **C.** Representative control (left) and heart failure (right) WGA stained (a) and skeletonized (b) images of ferret ventricular myocytes and t-tubule orientation analysis (c). **D.** Mean data showing that the ratio of transverse to longitudinal t-tubules does not change in pressure overload heart failure. Scale bars, 10 μm. ***P < 0.001; (Sheep, N = 6 control, 7 heart failure; Ferret, N = 4 control, 5 heart failure).**

**JPH2 and RyR colocalise in the absence of t-tubules**

We sought to understand the potential reasons for the constancy of JPH2 protein levels between sheep control and heart failure atrial myocytes and rat atrial and ventricular myocytes despite the respective loss and absence of t-tubules in these atrial tissues. As described above, JPH2 has been suggested to tether the RyR / SR membrane to t-tubules in order to maintain / stabilize dyadic architecture. However, as reported previously, and shown here (Online Figure VII.B.a.), rat atrial myocytes, which lack t-tubules, have a sarcomeric intracellular RyR distribution. We also show that in atrial myocytes isolated from sheep with tachypacing induced heart failure where t-tubules are lost (manuscript, fig 3 and 4), the RyR is also distributed regularly throughout the cell (Online Figure VII.A). Importantly, in both rat atrial and sheep heart failure atrial myocytes, JPH2 is also distributed throughout the cell and colocalises with the RyR indicating that JPH2 may serve to localize the RyR by binding to structures other than the t-tubule membrane. Qualitatively similar RyR and JPH2 colocalisation is present in rat ventricular myocytes (Online Figure VII.B.b.). Conversely, AmpII is largely absent or only patchily distributed in rat atrial cells and shows little or no colocalisation with WGA stained cell membranes (Online Figure VII.C.a.) whereas in rat ventricular tissue, where t-tubules are densely expressed, AmpII and WGA colocalise (Online Figure VII.C.b.).
Sheep Atria heart failure
A. RyR  JPH2  colocalised

Rat Atrial tissue
Ba) RyR  JPH2  colocalised

Rat Ventricular tissue
b) RyR  JPH2  colocalised

Rat Atrial tissue
Ca) WGA  AmpII  colocalised

Rat Ventricular tissue
b) RyR  JPH2  colocalised
**Online Figure VII. RyR, JPH2 and t-tubule distribution in atrial myocytes lacking t-tubules.**

A. Representative image (from 4 cells, 2 hearts) showing immunolocalisation of the RyR (red), JPH2 (green) and their colocalisation (yellow, Manders M1 = 0.95) in a sheep heart failure atrial myocyte. B. Immunolocalisation of the RyR (red), JPH2 (green) and their colocalisation (yellow) in rat atrial tissue (a; Manders M1 = 0.99; 15 cells, 4 hearts) and ventricular tissue (b, Manders M1 = 0.96; 6 cells, 2 hearts). C. Membrane staining with WGA (red) and immunolocalisation of AmpII (green) in representative rat atrial tissue sections showing minimal colocalisation (yellow) in atrial tissue (a, Manders M1 = 0.12; 9 cells, 3 hearts) and extensive colocalisation in ventricular tissue (b, Manders M1 = 0.92; 8 cells 2 hearts). Scale bars, 10 µm.

**Non-specific intracellular staining with WGA conjugates in permeabilised cells**

An important consideration when colocalising t-tubules (stained with WGA conjugates) and ion channels (e.g. LTCC) or proteins implicated in t-tubule formation and maintenance (e.g. AmpII or JPH2) is the possibility of non-specific WGA staining as a result of the cell permeabilisation step used during immunohistochemical processing. The possibility of such non-specific staining arises because WGA will bind to sialic acid and N-acetylglicosaminyl residues on any intracellular organelle once the cell has been permeabilised and is indicated to have occurred by the prominent nuclear staining noted in supplementary Online Figure V panels B & C. We have examined the possibility that the irregular intracellular staining observed in permeabilised cells (e.g. Online Figure V.C.i-ii.) results from non-specific staining rather than t-tubule staining by staining non-permeabilised and permeabilised rat atrial cells with WGA as shown in Online Figure VIII. It is clear that permeabilisation results in some intracellular WGA staining and therefore could explain the residual intracellular staining observed in the AmpII siRNA targeted cells when they are stained, following permeabilisation, with WGA conjugated AlexaFluor 488 (Online Figure V.C).

**Rat Atrial WGA Staining**

![Rat Atrial WGA Staining](image)

**Online Figure VIII. Cell Permeabilisation during immunohistochemical processing leads to non-specific intracellular staining with WGA conjugates.**

Images were obtained from isolated rat atrial myocytes and hence lack t-tubules. Cells were stained with WGA-AlexaFluor 488 conjugate without (left) or following fixation and membrane permeabilisation with ice-cold acetone (7 mins, centre and left panels).
It is clear that permeabilisation results in the appearance of WGA stained organelles within the cell including the nucleus (centre panel) and potentially golgi apparatus (centre and right panels). Scale bars 10 μm. Representative images displayed from 2 separate experiments.
References


