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# t-Tubule remodelling: a cellular pathology driven by both sides of the plasmalemma?

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**Abstract** Transverse (t)-tubules are invaginations of the plasma membrane that form a complex network of ducts, 200–400 nm in diameter depending on the animal species, that penetrates deep within the cardiac myocyte, where they facilitate a fast and synchronous contraction across the entire cell volume. There is now a large body of evidence in animal models and humans demonstrating that pathological distortion of the t-tubule structure has a causative role in the loss of myocyte contractility that underpins many forms of heart failure. Investigations into the molecular mechanisms of pathological t-tubule remodelling to date have focused on proteins residing in the intracellular aspect of t-tubule membrane that form linkages between the membrane and myocyte cytoskeleton. In this review, we shed light on the mechanisms of t-tubule remodelling which are not limited to the intracellular side. Our recent data have demonstrated that collagen is an integral part of the t-tubule network and that it increases within the tubules in heart failure, suggesting that a fibrotic mechanism could drive cardiac junctional remodelling. We examine the evidence that the linkages between the extracellular matrix, t-tubule membrane and cellular cytoskeleton should be considered as a whole when investigating the mechanisms of t-tubule pathology in the failing heart.

**Keywords** Transverse tubules · Excitation–contraction coupling · Heart failure · Extracellular matrix · Collagen

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## Introduction

To appreciate how transverse (t)-tubules aid a synchronous contraction and how their spatial remodelling cause a loss of function necessitates a biophysical understanding of the excitation–contraction coupling process that governs contraction. Myocyte contraction is initiated by electrical depolarisation of the plasma membrane (or sarcolemma) in the form of an action potential which allows an influx of extracellular $Ca^{2+}$ via the voltage-gated L-type $Ca^{2+}$ channel (LTCC) to trigger the intimately arranged ryanodine receptor (RyR) of the sarcoplasmic reticulum (SR). In a process called calcium-induced calcium release (CICR) (Bers 2002), this evokes the synchronised and rapid release of $Ca^{2+}$ at the flanks of every sarcomere of the myocyte. As free $Ca^{2+}$ rises in the cytosol, it binds to troponin C, on the contractile apparatus, causing a conformational shift that initiates cross-bridge cycling and drives contraction. Contraction is subsequently terminated by a decrease in cytosolic $Ca^{2+}$ concentration back to resting levels, predominately due to the action of SR calcium transport ATPase (SERCA), which pumps $Ca^{2+}$ back into the SR and, to a lesser extent, the sodium calcium exchanger (NCX) extruding $Ca^{2+}$ back into the extracellular space. NCX is crucial to ensuring that the initial trans-sarcolemmal $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels is transported back into the extracellular space to avoid cellular $Ca^{2+}$ accumulation over the contractile cycle. The t-tubules are an extension of the sarcolemma (Soeller and Cannell 1999) and provide a signalling pathway for the rapid propagation of the action potential deep within the myocyte interior, within milliseconds, facilitating a synchronous cell-wide $Ca^{2+}$ release that is much faster and larger than if the cell relied on $Ca^{2+}$ diffusion from initiation events at the cell surface (Kawai et al. 1999; Cordeiro et al. 2001; Brette et al. 2002, 2006; Louch et al. 2004; Sacconi et al. 2012). For example, a typical $Ca^{2+}$ transient in

rat myocytes with intact t-tubules reaches its peak $Ca^{2+}$ within ~60 milliseconds; if the t-tubules are removed by osmotic shock-induced detubulation with formamide, peak $Ca^{2+}$ is reached much later, in ~120 milliseconds (Crocini et al. 2014).

## Nanoscale structure of junctions: the template for local control of calcium signalling

A key structure of the CICR machinery is the cardiac junction or dyad where the plasma membrane (either at the sarcolemma surface or t-tubules) comes into close opposition, ~15 nm, with the sarcoplasmic reticulum (Forbes and Sperelakis 1982; Takeshima et al. 2000; Soeller and Cannell 2004). These structures form a coupling (couplon) between LTCC and RyR into cardiac 'synapse,' creating individual islands of $Ca^{2+}$ release. The couplons are essential for the graded response to increasing levels of trigger current. In other words, increased activation of LTCC increases the number of active couplons, increasing SR calcium release and the force of contraction. Conversely, decreased activation of LTCC decreases the number of active couplons, decreasing $Ca^{2+}$ release and contractile force. The mechanism by which the junction achieves this graded response was first described computationally by Stern (1992) and subsequently observed experimentally in confocal-line scan data of cytoplasmic $Ca^{2+}$, called '$Ca^{2+}$ sparks' (Cheng et al. 1993), which correspond to optically detectable events of $Ca^{2+}$ release from unitary junctions or functionally coupled junctions. The spatial separation of discrete $Ca^{2+}$ release sites throughout the cells prevents a positive feedback loop that would lead to an all-or-nothing release. As diffusional distances from the release site increase, $Ca^{2+}$ levels rapidly decline below the threshold required to initiate RyR opening, preventing the activation of adjacent release sites. For example, the average cytosolic free $Ca^{2+}$ ranges from 0.1 μm at diastole to 1 μm at systole, well below the >10 μm free $Ca^{2+}$ that is required to initiate RyR opening in single-channel recording experiments (Laver et al. 2013). Then how does the much smaller $Ca^{2+}$ current stimulate release? This is due to the narrow, diffusion-restricted space of the junction concentrating $Ca^{2+}$ from the LTCC to levels required to open the RyR (Cannell and Soeller 1997) . The clustered organisation of RyRs within the same junctional space, therefore, is expected to be under the same control of the local cytoplasmic (cleft) $Ca^{2+}$ concentration. The morphologies of RyR clustering within these nanoscale signalling sites also appear to be crucial, with recent simulations based on experimentally determined RyR arrays demonstrating that the fidelity of a spark being evoked by a localised LTCC opening is accentuated in larger clusters (Walker et al. 2014, 2015). Displacement of the crucial $Ca^{2+}$ handling proteins (LCC and NCX) of the junction in the order of tens of nanometres in computer simulations appears to be sufficient to disrupt the efficient opening of RyRs (Cannell et al. 2006; Sher et al. 2008). More recent microscopy work in atrial myocytes underscore that the mechanisms of local control of junctions are certainly not limited to the direct actions of $Ca^{2+}$; junctions may also provide a spatial compartment within which post-translational modification of RyRs (e.g. site-specific phosphorylations which alter RyR single-channel properties) can be optimised locally (Brandenburg et al. 2016).

## t-Tubule remodelling, local $Ca^{2+}$ release and heart failure

Evidence of defective CICR at the local scale in heart failure was initially observed in confocal line scan data from myocytes isolated from the salt-sensitive hypertensive rat (Gomez et al. 1997). The myocytes from these animals had a reduced number of calcium spark events despite a normal calcium current, indicating defective communication between the LTCC and RyR channels. Loss of t-tubules have been documented in a dog model of tachycardia-induced heart failure (Gomez et al. 1997; Balijepalli et al. 2003). Loss of both t-tubules and defective local $Ca^{2+}$ release (sparks) in heart failure was subsequently reported in the spontaneous hypertensive rat (Song et al. 2006) and myocardial infarction induced heart failure in mice (Louch et al. 2006) and pig (Heinzel et al. 2008). A consistent feature in these studies is the change of t-tubule orientation with loss of the transverse elements but increase in longitudinal elements. Both loss of t-tubule length and orientation could lead to a reduced number of couplons, as RyR clusters are predominantly transversely aligned with the Z-line in myocytes. In the spontaneous hypertensive rat myocytes, confocal microscopy demonstrated a loss of co-localisation of LTCC with RyR, suggesting that distorted dyad microarchitecture may also result in reduced local $Ca^{2+}$ release (Song et al. 2006). A similar loss of colocalisation has been observed in other animal models (Wu et al. 2011) and human heart failure (Crossman et al. 2011). Recently, the development of two-photon random access microscopy has allowed the simultaneous imaging of action potential and $Ca^{2+}$ release at t-tubules. This work has demonstrated, in myocytes from rats in which heart failure was induced by myocardial infarction, that there can be regions of apparently intact t-tubule that fail to propagate the action potential, resulting in delayed local $Ca^{2+}$ transient. Furthermore, in other t-tubular regions, spontaneous $Ca^{2+}$ release was found to trigger a local action potential (delayed after-depolarisation) that then, in turn, triggered a larger $Ca^{2+}$ release in the same region, indicating a role of t-tubules in arrhythmic events common in heart failure (Crocini et al. 2014). To what extent these different mechanisms arising from t-tubule remodelling contribute to the development of heart failure remains to be elucidated.

However, in general, t-tubule remodelling appears to contribute mechanistically to the loss of contractility found in heart failure (Brette and Orchard 2007). For example, confocal imaging of the intact living hearts in thoracic aortic banded rats (model of pressure overload) demonstrated a progressive remodelling and loss of t-tubules as animals transitioned from hypertrophy to chronic heart failure that was strongly correlated to loss of ejection fraction (Wei et al. 2010).

Disrupted local $Ca^{2+}$ signalling has been previously documented in the myocytes from the failing human heart (Beuckelmann et al. 1992; Lindner et al. 2002; Louch et al. 2004). However, there have been conflicting reports on t-tubule remodelling in the failing human heart. An early electron microscopy (EM) study of hypertrophic cardiomyopathy tissue found loss or absence of t-tubules, which were irregularly shaped and often dilated (Maron et al. 1975). However, subsequent studies (including EM and confocal microscopy) showed proliferation and dilation of t-tubules in both dilated and ischaemic cardiomyopathy tissue (Schaper et al. 1991; Kaprielian et al. 2000), whereas a subsequent confocal study showed a loss and dilation of t-tubules in dilated cardiomyopathy tissue (Kostin et al. 1998). A study using isolated myocytes and two-photon microscopy found no change in t-tubular volume in failing hearts of different aetiologies; dilated, ischemic and familial hypertrophic cardiomyopathy (Ohler et al. 2009). In contrast, a study of isolated myocytes analysed with high-resolution scanning ion-conductance microscopy demonstrated a loss of t-tubule openings on the cell surface in similar aetiologies; ischemic, dilated and hypertrophic cardiomyopathy (Lyon et al. 2009). In our confocal analysis of dilated cardiomyopathy tissue, in agreement with previous reports, t-tubule diameter was substantially increased in heart failure. However, we found a wide variability of t-tubule structure between myocytes: some cells had near-normal t-tubule abundance and morphology, some cells largely lacked t-tubules, some cells were dominated by longitudinal t-tubule elements and yet other cells were dominated by oblique running t-tubules that would cross several sarcomeres. Analysis of the t-tubule angle showed a shift from the dominance of transversely oriented tubules in non-failing hearts to oblique and longitudinal angles in failing hearts (Crossman et al. 2011), similar to changes observed in animal models. Suspecting that regional differences in cardiac function previously documented in dilated cardiomyopathy (Young et al. 2001) may explain the variability in t-tubule structure, we undertook a regional cardiac magnetic resonance imaging strain analysis of dilated cardiomyopathy patients. These tissue regions were subsequently biopsied at transplant and analysed by confocal microscopy. This analysis demonstrated a strong correlation between t-tubule structure and contractility, with regions having near-normal contractility having largely intact t-tubules, whereas regions with poor contractility had lost much of the transverse elements (Crossman et al. 2015). These data offer a possible explanation to conflicting reports on t-tubule remodelling in human heart failure that the variability was due to unknowingly sampling regions of differing contractile function. Interestingly, a recent EM study showed a marked remodelling of junctional structure in both dilated cardiomyopathy and ischaemic heart failure (Zhang et al. 2013). The junctional structure will likely also be impacted by structural remodelling of the SR in heart failure (Pinali et al. 2013). The consensus opinion based on the above studies and those on animal models is that remodelling of t-tubules is an important feature in the pathology of many forms of heart failure (Guo et al. 2013).

## Mechanisms of t-tubules remodelling

The mechanisms that drive t-tubule remodelling are poorly understood and are an area of active research. It has been argued that the local mechanical environment or mechanical stress that myocytes experience plays an important role in t-tubule dynamics (Ibrahim and Terracciano 2013). For example, isolated rat cardiac myocytes, which are digested from the extracellular matrix (ECM) and are mechanically unloaded, lose their t-tubules within 24 to 48 h in culture (Louch et al. 2004). Conversely, too much stress as experienced in heart failure appears to be a driver for pathological remodelling; for example, high-stress regions near infarct sites typically have a low abundance of t-tubules (Frisk et al. 2016). Direct evidence for this mechanism was demonstrated by mechanical unloading of the ischaemic failing heart in the rat by heterotopic abdominal heart transplantation; this procedure reversed t-tubule remodelling and normalised local $Ca^{2+}$ release (Ibrahim et al. 2012). The same procedure used for prolonged mechanical unloading of normal hearts led to loss of t-tubule structure and impaired $Ca^{2+}$ signalling (Ibrahim et al. 2010), indicating that there is a 'Goldilocks zone' of mechanical load. The synchronisation between myocytes also appears to be important, as dyssynchronous heart failure in the dog is characterised by loss of t-tubule structure and impaired calcium release that can be reversed by cardiac resynchronisation therapy (Sachse et al. 2012; Li et al. 2015). So how can organ level load affect remodelling of t-tubules at the sub-cellular scale? For the heart to achieve its critical pump function, it is necessary for individual myocytes to work together as a syncytium. In other words, millions of individual myocytes are connected together in a manner that the contractile forces generated by each individual cell are coordinated into a tightly synchronised organ-level contraction required for an effective mechanical pump. When the heart experiences mechanical overload or dyssynchrony, these forces are presumably transmitted back to the individual cells. Consistent with this proposition is that mechanical strain applied to isolated living rabbit myocytes leads to dynamic changes in t-tubule shape

(McNary et al. 2011). Whilst there are no mechanistic data that we know of which *directly* evaluate an intrinsic mechanosensing capability of t-tubules, its communication with the stretch-sensitive Z-disc telethonin (Tcap) appears to help regulate t-tubule morphology. Knockout of this Tcap leads to t-tubule remodelling and disrupted $Ca^{2+}$ release, which is exacerbated by overload induced by the thoracic aortic banding model of heart failure (Ibrahim et al. 2013). It is conceivable then that aberrant chronic load leads to pathological distortion of t-tubules and junctional structure, leading to miscommunication between the LTCC and RYR receptors.

At the molecular scale, several candidate proteins have been identified that are required for normal junctional structure and appear to be involved in t-tubule remodelling in heart failure (van Oort et al. 2011; Zhang et al. 2014; Hong et al. 2014; Wu et al. 2014; Guo et al. 2014; Caldwell et al. 2014). The first of these proteins identified was junctophilin-2 (JPH2), a protein that forms a connection between the plasma and SR membranes. Knockout of this protein in mice was found to be embryonically lethal, with hearts having abnormal junctional structure, and diminished and irregular $Ca^{2+}$ transients (Takeshima et al. 2000). A later studied found that mutations in JPH2 were associated with hypertrophic cardiomyopathy in humans (Landstrom et al. 2007). Subsequent inducible and cardiac-specific knockdown of JPH2 in adult mice resulted in loss of contractility, heart failure and increased mortality that was associated with disrupted $Ca^{2+}$ release and reduced LTCC and RyR colocalisation (van Oort et al. 2011). Another protein found to be critical to junctional structure is the membrane scaffolding protein BIN-1. Knockout of BIN-1 is prenatally lethal, with embryos displaying severe cardiomyopathy in EM (Muller et al. 2003). Subsequent investigation in adult mice with immunocytochemistry, EM and immunoprecipitation revealed that BIN-1 was required for trafficking LTCC into t-tubules via the cellular microtubule network. Transient knockdown in mice cardiac myocytes reduced surface levels of LTCC and delayed the $Ca^{2+}$ transient (Hong et al. 2010). Furthermore, cardiac-specific BIN-1 knockout leads to loss of dense membrane folds in the mouse, promoting susceptibility to ventricular arrhythmia (Hong et al. 2014). Microtubule densification, a known feature of heart failure, was later linked to defective JPH2 trafficking and t-tubule remodelling (Zhang et al. 2014). Loss of JPH2 and t-tubule remodelling has also been linked to overactivation of heterotrimeric G protein Gαq that occurs in cardiac hypertrophy (Wu et al. 2014; Huang et al. 2016). Recently, a novel junctional protein, striated muscle preferentially expressed protein kinase (SPEG), has been identified and its knockout in mice, leading to t-tubule remodelling, aberrant local $Ca^{2+}$ handling and heart failure (Quick et al. 2016). Yet another protein linked to t-tubule morphology is caveolin-3 (cav-3), a protein involved in the formation of caveolae, small (50–100-nm) membrane invaginations (Galbiati et al. 2001). Knockout of cav-3 in mice leads to abnormalities in the skeletal muscle t-tubule structure and exclusion of the dystrophin–glycoprotein complex (DGC) from lipid raft domains. Mutations in cav-3 are also associated with a form of limb-girdle muscular dystrophy (Minetti et al. 1998). Several of these proteins [e.g. JPH2 (Zhang et al. 2013), BIN-1 (Hong et al. 2012), SPEG (Quick et al. 2016) can be down-regulated in human heart failure but their relative contribution has yet to be determined. It is also likely that there is a complex interaction between these proteins and placing these interactions into a broader cellular and tissue context will be required to understand the process of t-tubule remodelling.

## Costameres, collagen and t-tubule remodelling

The finding of an interaction between proteins involved in t-tubule remodelling and the cytoskeleton, particularly the DGC, brings us to the next topic: the costamere complex and its interaction with the ECM. The costamere is a Z-disc-associated sub-plasma membrane complex that physically couples the force-generating sarcomeres to the sarcolemma and ECM (Peter et al. 2011). This complex has been described as the 'Achilles heel of striated muscle' due to its involvement in muscular dystrophies and cardiomyopathies (Ervasti 2003). Two major costamere protein assemblies have been identified: the DGC and the vinculin–talin–integrin system. These complexes are found within the cardiac t-tubules (see Fig. 2), which are located at the Z-disc in cardiac muscle, and are thought to provide mechanical stability to the t-tubules during contraction (Kostin et al. 1998). In addition to the costameres' role in muscle force production, they are also signalling domains that convert mechanical stimuli to biochemical signals (Muller et al. 2003; Lyon et al. 2015). Given this mechanotransduction role, the costamere is a potential source of aberrant signalling that drives t-tubule remodelling in heart failure.

The initial clue that led us to consider the costamere complexes and its interaction with the ECM in t-tubule remodelling came from our finding of enlargement and disarrangement of t-tubules in the failing human heart using confocal imaging of tissue sections labelled with wheat germ agglutinin (WGA) (Crossman et al. 2011, 2015). WGA is a plant lectin that selectively binds to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues of glycoconjugates of protein and lipid (Wright 1984). This label is commonly used in fluorescence microscopy to visualise the cell membrane, including the visualising of t-tubules in cardiac myocytes (Stegemann et al. 1990; Laflamme and Becker 1999; Balijepalli et al. 2003; Savio-Galimberti et al. 2008; Richards et al. 2011; Glukhov et al. 2015; Li et al. 2015). In addition to its membrane association, it also has extensive ECM labelling in human heart tissue (Crossman et al. 2011,

2015), see Fig. 1, and appears to bind to collagen in histological sections of skin and heart (Söderström 1987; Emde et al. 2014). Furthermore, WGA chromatography has been used to isolate the dystrophin glycoprotein complex from muscle (Campbell and Kahl 1989). This complex is critical to normal muscle function, with mutations in the associated genes commonly leading to muscular dystrophy and to heart failure in older patients (Lapidos et al. 2004; Verhaert et al. 2011). Moreover, changes in dystrophin labelling have been observed in end-stage human heart failure (Vatta et al. 2002) and dystrophin remodelling is associated with hypertrophied t-tubules in the failing human heart (Kaprielian et al. 2000). This led us to hypothesise that increases in one or more members of the DGC could be responsible for the observed increased WGA labelling and dilation of t-tubules in heart failure.

To identify which glycoproteins are bound by WGA, protein blots of human heart were probed with fluorescently labelled WGA (Crossman et al. 2017) (Fig. 2). This analysis highlighted a 5.7-fold increase in a 140-kDa WGA-positive band in heart failure. This band was subsequently analysed by protein fingerprinting using mass spectrometry and identified collagen VI as a likely candidate. Western blotting confirmed its identity as collagen VI and demonstrated a 2.4-fold increase in heart failure. Pertinently, mutations in collagen VI can result in Ullrich congenital muscular dystrophy and Bethlam myopathy (Lapidos et al. 2004), suggesting the important role of this collagen in muscle function. Confocal microscopy demonstrated colocalisation of collagen VI and dystrophin at t-tubules. However, a higher resolution method was required to resolve the distribution of these proteins around the small diameter t-tubules, which can be small as ~50 nm in diameter (Soeller and Cannell 1999). This was achieved with a super-resolution microscopy method that produces 30-nm resolution images using conventional fluorescent dyes (Heilemann et al. 2008; Baddeley et al. 2009, 2011). This technique is a form of localisation microscopy where only a few but highly resolved fluorophores are imaged at any one time. These molecules can then be localised to a precision of ~15 nm. By controlling the photochemistry, different and random subsets of fluorophores are switched on and off and their positions recorded. During an imaging run, tens of thousands of fluorophores are localised; from these data, a high-resolution fluorescent image is generated. Super-resolution imaging of human heart tissue clearly revealed that collagen VI was within the t-tubular lumen and that collagen bundle diameters increased in heart failure (Fig. 2). Assessment of tissue levels of collagen VI showed a change of distribution from a predominately basement membrane labelling pattern to one dominated by increased interstitial labelling reminiscent of fibrillar collagen fibrosis. Subsequent, confocal and super-resolution microscopy work then identified increased fibrillar collagens, type I and III, within the t-tubular lumen in heart failure (Crossman et al. 2017). These data are highly suggestive that increases of collagen could be involved in the aberrant t-tubule remodelling that occurs in heart failure.

## Collagens and nanoscale t-tubule remodelling

Collagen provides a unifying mechanistic link between organ-level changes (increases in load or dyssynchronous contraction) that could drive t-tubule remodelling in heart failure, particularly as changes in mechanical load, including cyclical load, influences collagen synthesis (Carver et al. 1991; Bishop and Lindahl 1999; Humphrey et al. 2014). We also note that the loss of t-tubules in cultured cardiac myocytes could be related to loss of collagen, as collagenases are used to isolate cells from the heart before culturing. In heart failure, fibroblasts are the source of increases of fibrillar collagen that

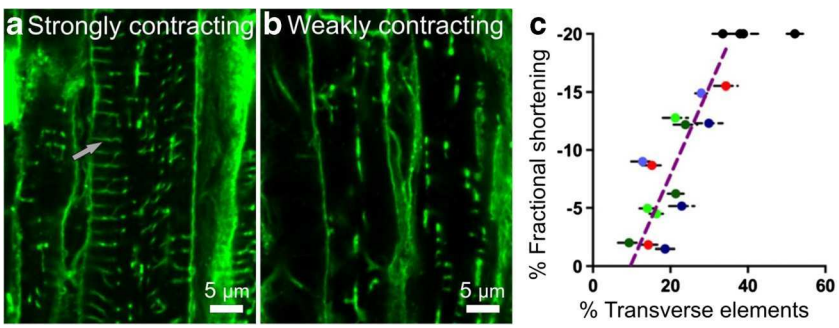


**Fig. 1** Loss of contractility in human heart failure is strongly correlated to loss of the transverse elements of the t-system. **a** and **b** show exemplar confocal micrographs of WGA-stained t-tubules from the strongly contracting region (−12% fractional shortening) and the weakly contracting region (−2% fractional shortening) in a failing heart. The *grey arrow* in **a** indicates a t-tubule. Note the loss of the transverse elements of the t-system in the poorly contracting tissue region. **c** demonstrates the strong correlation ($p < 0.001$) between the percent transverse elements (of the t-system) and fractional shortening of failing heart tissue regions. Five failing hearts were analysed, with *coloured circles* of the same colour indicating different regions from the same heart. Note how the trend is consistent within the individual failing hearts. The *black circles* are normal donor hearts shown for comparison and are not part of the regression analysis. Figure adapted from Crossman et al. (2015)

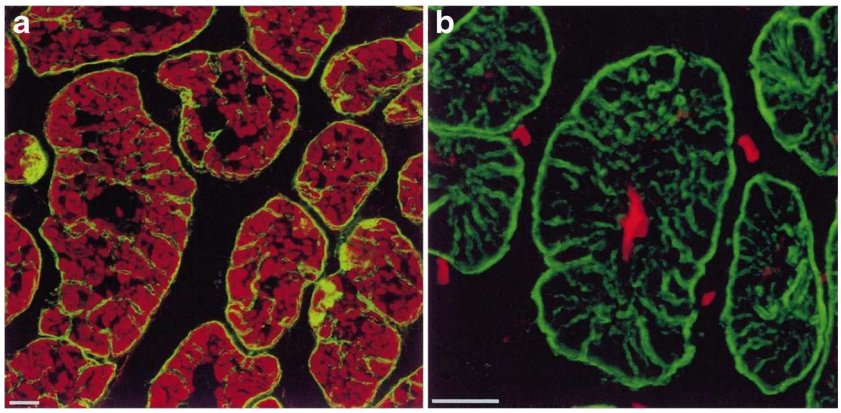


**Fig. 2** Costamere complexes are present within the t-tubules of the human heart. **a** Vinculin (*green*) labels both the surface and t-tubule sarcolemma, seen as radial finger-like projections situated between myofibrils, labelled with TRITC-conjugated phalloidin (*red*). **b** Dystrophin (*green*) labels both the surface and t-tubule sarcolemma. Nuclei are stained red with 7-AAD. Scale bars = 10 μm. Figure adapted from Kostin et al. (1998)

characterises fibrosis (Segura et al. 2014), indicating that fibroblasts could be involved in t-tubule remodelling. Fibroblasts are also the likely source of collagen VI, as evidenced by the increased numbers of collagen VI-positive fibroblasts we found in human dilated heart failure (Crossman et al. 2017) and is in agreement with a cell culture study of skeletal muscle that only found collagen VI protein and mRNA in fibroblasts and not in other cell types (Zou et al. 2008). Particularly interesting is the identification of collagen VI-positive fibroblast filopodia within the lumen of some of the enlarged t-tubules in heart failure, supporting a likely role of fibroblasts in either maintaining or remodelling t-tubules from within the tubule lumen.

The linkage between costamere complexes and the ECM, particularly the collagens, indicate a mechanism, which, by either direct mechanical interaction or signalling (mecha notransduction), under extremes of load (high or low), could lead to disrupted junctional complexes. For example, collagen VI is likely a part of the dystrophin complex, because mutations in both proteins result in muscular dystrophy (Lapidos et al. 2004; Allamand et al. 2011), WGA binds collagen VI (Fig. 2) and WGA chromatography has been used to isolate dystrophin through binding to unknown component of the DGC (Campbell and Kahl 1989), which we suggest is collagen VI. In addition, the protein biglycan can bind to both collagen VI and the DGC (Wiberg et al. 2001, 2002; Rafii et al. 2006). Dystrophin connects the sarcolemma to cellular cytoskeleton components, including actin filaments and microtubules (Renley et al. 1998; Prins et al. 2009), which, in turn, link to the L-type $Ca^{2+}$ channel (Viola et al. 2014). Furthermore, microtubules have been implicated in trafficking both LTTC and RyR to cardiac junctions (Hong et al. 2010; Zhang et al. 2014). Potentially, the increased dilation of t-tubules on the order of ~150 nm could lead to structural remodelling of the cellular cytoskeleton, leading to the displacement of LTTC and RyR in cardiac junctions on the order of 10–20 nm required to disrupt $Ca^{2+}$ release. Consistent with this proposal, the MDX mouse that has no dystrophin has disrupted cellular cytoskeleton (Viola et al. 2014) and disrupted calcium release (Cheng et al. 2012). In addition, the vinculin–talin–integrin system could likely have a role in t-tubule remodelling, given its linkage to the actin cytoskeleton (Ziegler et al. 2008) and the promiscuity of integrin (depending on the receptor sub-type) to bind to different ECM components, including laminin, fibronectin and collagens, including types I, III, IV and VI (Ross and Borg 2001; Tulla et al. 2001), which have all been shown to be within the t-tubules (Kostin et al. 1998; Crossman et al. 2017). These observations highlight that any number of ECM proteins could participate in either mechanical or chemical communications which could determine the local t-tubule structure.

## Future directions

The data linking collagen to t-tubule remodelling were obtained in samples of end-stage human heart failure. In order to establish collagen as a mechanism of t-tubule remodelling, the time course of changes in collagens, t-tubular structure, $Ca^{2+}$ handling and contractile function, in response to a suitable intervention, must be considered together. This would almost certainly require, in the medium term, the use of a suitable animal model and begs the question what animal model would be most appropriate. As noted above, changes in the t-tubular structure in animals models mimic the change in orientation from dominance in the transverse direction in normal myocytes to dominance of the longitudinal direction in failing myocytes that is characteristic of the failing human heart. However, a consistent feature of human heart failure is the increased dilation of t-tubules, which we suggest is a consequence of increased collagen deposition. The dilation of the t-tubules in animal models of heart failure has remained uncharacterised until relatively recently (Wagner et al. 2012). This likely reflects the smaller diameter of t-tubules in rodents that are commonly used to study t-tubule remodelling in heart failure. For example, rodent t-tubules are ~200 nm in diameter, whereas in humans, the diameter is ~400 nm. Critically, this difference in diameter is at the

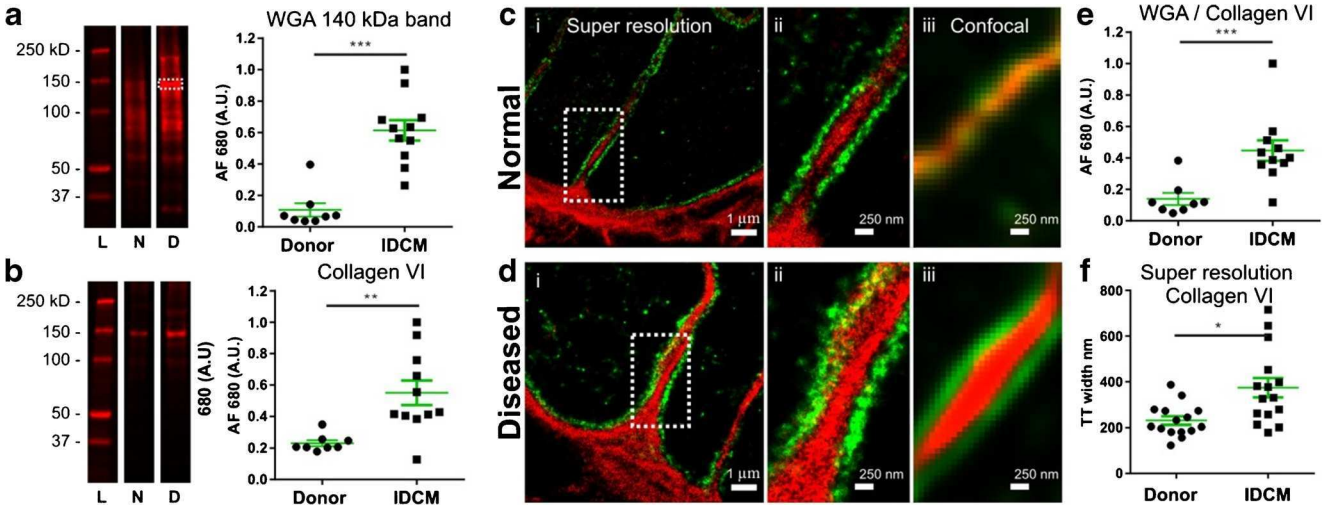


**Fig. 3** t-Tubules are dilated in myocytes from the coronary artery ligation rat model of myocardial ischaemic heart failure. Sections of rat heart were labelled with WGA-488 and imaged on a Zeiss LSM 880 Airyscan with 63 × 1.4 NA oil lens. **a** Exemplar of t-tubule labelling of a myocyte from a sham operated animal. **b** Exemplar of t-tubule labelling in myocyte from an ischaemic heart failure (*MI*) animal. **c** The frequency distribution of the full width at half maximum (FWHM) of the transverse component of t-tubules were measured in 150 sham t-tubules from five hearts and 150 MI t-tubules from five hearts. The mean diameter of WGA t-tubules in sham myocytes was 222 ± 3 nm and was significantly smaller compared to those in MI myocytes, being 253 ± 4 nm (*$p$ = 0.02; two-factor nested ANOVA)

boundary of the optical resolution limit of ~250 nm for the confocal systems commonly used to image t-tubules and has likely led to the obscuring of this feature of pathology in rodent models of heart failure.

The advent of super-resolution microscopy is resolving this issue. For example, stimulated emission depletion microscopy, with ~50-nm resolution, has been developed for live cell imaging of isolated myocytes (Wagner et al. 2012). This study examined myocytes from a coronary artery ligation mouse model of ischaemic heart failure, demonstrating a significant increase in t-tubule diameter from 197 nm in sham animals to 216 nm in heart failure animals. Recently, we have used Airyscan confocal microscopy, which has 1.7-fold improvements in resolution over the standard (pinhole-based confocal) method (Huff 2015), to examine the WGA-labelled t-tubules in a coronary artery ligation rat model of ischaemic heart failure. This analysis, presented in Fig. 3, demonstrates not only a disrupted t-tubule organisation but significantly increased diameter of t-tubules in the failing heart, consistent with findings in human and mouse heart failure. However, it should be noted that the increases in diameter of mouse and rat t-tubules in heart failure (10% and 15%, respectively) are relatively modest compared to the t-tubule diameter change from 294 nm to 436 nm (or 48% increase) that occurs in human heart failure (Crossman et al. 2017). The smaller diameter of t-tubules in rodents may restrict the extent that collagen can accumulate within the lumen. Potentially, species with larger diameter t-tubules may provide a more physiologically relevant model for the study of t-tubule remodelling. For example, the healthy rabbit has an average t-tubule diameter of 356 nm (Kong et al. 2017). The rapid rise in the popularity and availability of commercial instruments which circumvent the resolution limit of conventional optical microscopy methods will open access to this technology to many cardiac researchers and will likely play a key role in understanding the mechanisms of t-tubule remodelling in future heart failure studies. For a detailed discussion of super-resolution instrumentation and how it relates to cardiac research, the reader is referred to the following review articles: Kohl et al. (2013), Soeller and Baddeley (2013) and Jayasinghe et al. (2015).

Recent studies in rodents also indicate that collagen, particularly collagen VI, could have an important role in the pathogenesis of t-tubule remodelling in ischaemic heart failure. Mice with knockout of the collagen VI gene were paradoxically protected from the development of heart failure after coronary ligation induction of myocardial infarction. This was demonstrated by improved ejection fraction and reduced infarct size relative to wild-type controls (Luther et al. 2012). The t-tubule structure was not assessed in this study but we suspect that this functional improvement, at least partially, could be due to the prevention of t-tubule remodelling. Furthermore, t-tubule remodelling is reduced using beta blockade in ischaemic heart failure in mice (Chen et al. 2012), a treatment that is known to mitigate fibrosis in ischaemic heart failure in rats (Wei et al. 2000). In general, there is a paucity of data on the role of ECM and t-tubule dynamics. Future studies will need to determine which collagens (or other ECM proteins) are involved and the how the time frame of changes occurs in relation to disease development to identify optimal times for therapeutic intervention. Ischaemic patients generally present early after a myocardial infarction, before progression to heart failure, offering a therapeutic

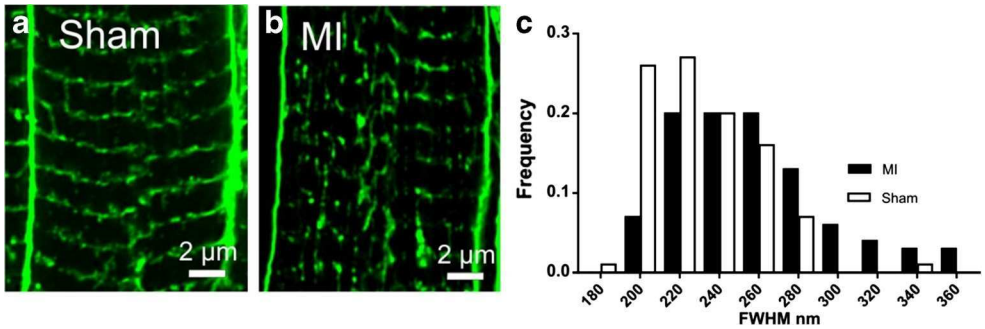


**Fig. 4** Increased WGA labelling of t-tubules is due to collagen VI in idiopathic dilated cardiomyopathy in humans. **a** WGA Western blotting identifies an increase in the 140-kDa band in disease. **b** Western blotting (and mass spectrometry) identifies this band as collagen VI. **c, i** and **d, i** Super-resolution microscopy of normal and diseased t-tubules labelled for collagen VI (*red*) and dystrophin (*green*). **c, ii** and **d, ii** Zoom of box in **i**. **c, iii** and **d, iii** Confocal microscopy of equivalent t-tubules. **e** t-Tubule diameter measured from collagen VI labelling. **f** Ratio of WGA intensity over collagen VI intensity. L = ladder, N = normal, D = heart failure. For Western blotting, $n = 7$ normal and 11 diseased hearts. For super-resolution microscopy, $n = 15$ t-tubules from five normal hearts and 15 t-tubules from five diseased hearts. $^{***}p < 0.001$. $^{**}p < 0.01$. $^{*}p < 0.05$. Figure adapted from Crossman et al. (2017)

window prior to the development of chronic disease. For this reason, effective preventative therapies will likely have the most impact for ischaemic heart failure. Targeted therapies could include some of the newer anti-fibrotic drugs, such as pirfenidone, that block collagen I synthesis in human lung fibroblasts (Nakayama et al. 2008) and can reduce fibrosis in ischaemic heart failure in rats (Nguyen et al. 2010). Furthermore, the data in Fig. 4 indicate increased glycosylation of collagen VI in heart failure (due to the greater increase in WGA binding relative to the smaller increase in the amount of collagen VI protein). Importantly, glycosylation of hydroxylysines is essential for the function of collagen VI (Sipilä et al. 2007). Although the significance of this increased WGA binding is not presently known, if it was linked to t-tubule remodelling, it could potentially be targeted.

## Conclusion

The recent finding of increased collagen within the dilated transverse (t)-tubules in the failing human heart raises the intriguing possibility that fibrosis at the nanoscale could drive t-tubule pathology. This hypothesis provides a unifying mechanism that directly links pathological increases in mechanical load at the organ and tissue levels with nanoscale structural changes that are thought to impair $Ca^{2+}$ release in the failing heart. The advent of super-resolution imaging technologies now provides the tool with which to critically test this new hypothesis. However, to maximise the benefit from the increased resolution, the nanoscale data need to be contextualised within the multi-scale levels of organisation found within the heart, including molecules, organelles, cells (myocytes and fibroblasts), extracellular matrix and tissue regions within the whole organ.

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**Compliance with ethical standards**

**Conflict of interest** David J. Crossman declares that he has no conflict of interest. Isuru D. Jayasinghe declares that he has no conflict of interest. Christian Soeller declares that he has no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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