# Mechanical unloading reverses transverse tubule remodelling and normalizes local Ca$^{2+}$-induced Ca$^{2+}$ release in a rodent model of heart failure

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| **Aims** | Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) is critical for contraction in cardiomyocytes. The transverse (t)-tubule system guarantees the proximity of the triggers for Ca$^{2+}$ release [L-type Ca$^{2+}$ channel, dihydropyridine receptors (DHPRs)] and the sarcoplasmic reticulum Ca$^{2+}$ release channels [ryanodine receptors (RyRs)]. Transverse tubule disruption occurs early in heart failure (HF). Clinical studies of left ventricular assist devices in HF indicate that mechanical unloading induces reverse remodelling. We hypothesize that unloading of failing hearts normalizes t-tubule structure and improves CICR. |
| **Methods and results** | Heart failure was induced in Lewis rats by left coronary artery ligation for 12 weeks; sham-operated animals were used as controls. Failing hearts were mechanically unloaded for 4 weeks by heterotopic abdominal heart transplantation (HF-UN). HF reduced the t-tubule density measured by di-8-ANEPPS staining in isolated left ventricular myocytes, and this was reversed by unloading. The deterioration in the regularity of the t-tubule system in HF was also reversed in HF-UN. Scanning ion conductance microscopy showed the reappearance of normal surface striations in HF-UN. Electron microscopy revealed recovery of normal t-tubule microarchitecture in HF-UN. L-type Ca$^{2+}$ current density, measured using whole-cell patch clamping, was reduced in HF but unaffected by unloading. The variance of the time-to-peak of the Ca$^{2+}$ transient, an index of CICR dyssynchrony, was increased in HF and normalized by unloading. The increased Ca$^{2+}$ spark frequency observed in HF was reduced in HF-UN. These results could be explained by the recoupling of orphaned RyRs in HF, as indicated by immunofluorescence. |
| **Conclusions** | Our data show that mechanical unloading of the failing heart reverses the pathological remodelling of the t-tubule system and improves CICR. |
| **Keywords** | HF • Left ventricular assist device • Recovery |

## Introduction

The transverse (t)-tubules of ventricular cardiomyocytes are an extensive system of membrane invaginations which are critical to Ca$^{2+}$-induced Ca$^{2+}$ release (CICR), and therefore to cellular contractility.[1,2] The efficiency of CICR is largely determined by the proximity of the Ca$^{2+}$ release trigger [dihydropyridine receptors (DHPR)] to the sarcoplasmic reticulum (SR) Ca$^{2+}$ release channels [ryanodine receptors (RyRs)].[2] As the DHPRs reside in the t-tubules, this proximity depends on normal t-tubule structure.

The t-tubules are disrupted in mechanical overload[3] and heart failure (HF).[4] T-tubule disruption is common to HF irrespective

of aetiology and species.[2,5] T-tubule defects and aberrant $Ca^{2+}$ handling are spatially co-localized, and may be mechanistically linked.[2,3,6,7] T-tubule disruption uncouples the normally tight interaction between DHPRs and RyRs.[3] Such changes occur early in the progression from hypertrophy to HF, suggesting that they may be important drivers of dysfunction.[8] The t-tubule system appears to be specifically load sensitive,[9] as evidenced by changes during mechanical overload[8] and unloading.[10]

Heart failure remains a serious clinical issue with a poor prognosis.[11] Left ventricular assist device (LVAD) therapy is used to sustain the circulation of patients in end-stage HF in whom they can induce myocardial recovery.[12–14] However, clinical trials suggest that mechanical unloading induces initial functional improvements in the native heart which regress over time.[15] Prolonged mechanical unloading of normal hearts distorts the t-tubules, resulting in dysfunctional $Ca^{2+}$ handling.[10] However, it is possible that in cardiac tissue subjected to chronic overload, such as in failing hearts, the disruption of the t-tubule system regresses with normalization of the load during mechanical unloading.

To test the hypothesis that t-tubule dysfunction of HF is reversible by mechanical unloading (HF-UN) and that this improves cellular $Ca^{2+}$ handling, we studied the structural and functional properties of failing rat myocytes 4 weeks after heterotopic abdominal heart transplantation.

# Methods

Extensive details may be found in the Supplementary material online. Syngeneic male Lewis rats (12 animals in total, 10–12 weeks old, ~220 g) were used in all experiments. All animal procedures were approved by the UK Home Office. Four animals were used in each group.
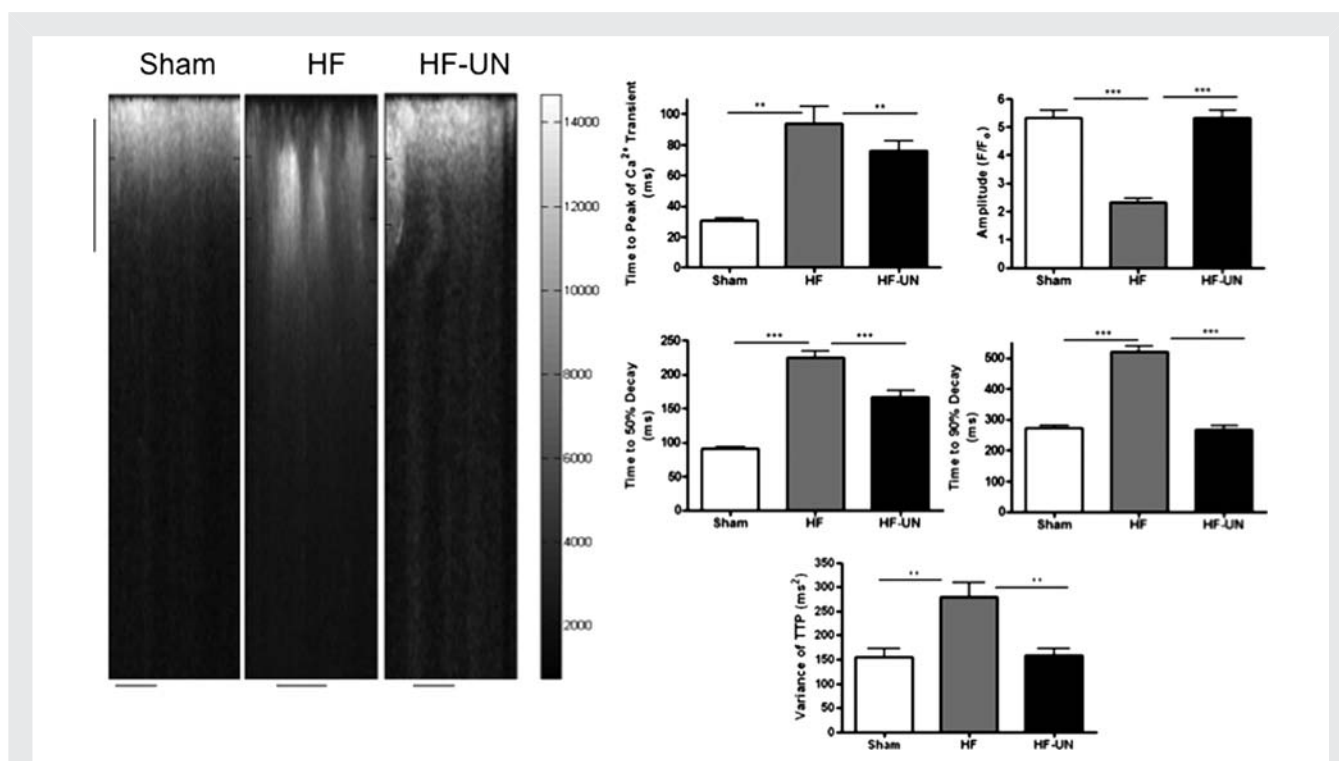
## Heart failure model

Heart failure was defined as an ejection fraction of <40% (measured by echocardiography) 12 weeks after left coronary artery ligation (see Supplementary material online). Sham-operated animals were used as controls.

## Mechanical unloading model

After the failing heart was harvested from the thorax, it was heterotopically transplanted into the abdomen of an age-matched syngeneic recipient as described previously[16] for 4 weeks.

## Cell isolation

Cardiomyocytes were isolated by standard enzymatic digestion only from non-infarcted left ventricular (LV) tissue as described previously.[17] All recordings were performed with cells superfused with normal Tyrode's solution (140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with 2 M NaOH) unless otherwise indicated.



**Figure 1** Mechanical unloading (HF-UN) induces a normalization of $Ca^{2+}$ transient parameters. The time-to-peak, decay to 50%, and decay to 90% of the $Ca^{2+}$ transient were prolonged in heart failure (HF). Mechanical unloading normalized these parameters. The amplitude of the $Ca^{2+}$ transient was depressed in HF and normalized by mechanical unloading. The variance of the time-to-peak, an index of $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) synchronicity, was increased in HF and normalized by mechanical unloading. The vertical scale bar indicates 200 ms, and the horizontal scale bar indicates 100 pixels (sham $n = 40$, HF $n = 34$, HF-UN $n = 29$).

## $Ca^{2+}$ handling imaging and electrophysiology

Fluo-4 AM and confocal microscopy were used to record $Ca^{2+}$ transients and sparks (diastolic $Ca^{2+}$ release events). The scale alongside line scans shown in *Figure 1* are greyscale charts of the fluorescence range, normalized to the background. Indo-1 was used to assay SR $Ca^{2+}$ content as previously described.[18,19] Cells were field stimulated at 1 Hz to obtain steady-state contractions.

L-type $Ca^{2+}$ current was measured in voltage-clamp mode as described previously.[20]

## Imaging of transverse tubules

Cells were stained with di-8-ANEPPS (Molecular Probes, OR, USA) and imaged using confocal microscopy.[10] During the Fourier analysis to assess t-tubule regularity, a central portion of the high resolution image of the cell was analysed and was always of fixed dimensions. To avoid bias, cells were codified and blinded. Imaging of cell surface topography was performed using scanning ion conductance microscopy (SICM). For electron microscopy, isolated cardiomyocytes were attached to coverslips using a Shandon Cytospin 2® centrifuge and then digital micrographs were taken using Gatan digital micrograph software at × 30 000 magnification and analysed by measuring the maximum t-tubule diameter of transverse t-tubules, the number of t-tubules per optical section.

## Protein subcellular localization and western blotting

Co-localization of DHPRs and RyRs was determined using immuno-fluorescence on isolated, fixed (with cold acetone) cardiomyocytes. Mouse anti-DHPR alpha 1 (Abcam, Cambridge, UK) antibodies were used against DHPRs, and rabbit anti-RyR (Santa Cruz Biotechnology

Inc., Santa Cruz, CA, USA) was used against RyRs. The percentage overlap between signals was calculated as an index of co-localization. Standard western blotting was performed to measure the expression of junctophilin-2 (JP-2; junctophilin-2 rabbit polyclonal antibody, Life-Span Biosciences LS-C82883).

## Statistical analysis

Statistical analysis was performed using non-parametric one-way analysis of variance (ANOVA; Kruskall−Wallis test). Dunn's post-hoc test was used to test for differences between groups. The analysis was performed using Prism 4 software (GraphPad software Inc., San Diego, CA, USA). $P < 0.05$ was taken as significant.

# Results

## $Ca^{2+}$ transient features are improved after mechanical unloading

Heart failure cells showed a depressed $Ca^{2+}$ transient amplitude, which improved in HF-UN. An important determinant of the $Ca^{2+}$ transient amplitude is SR $Ca^{2+}$ content, which was depressed in HF and increased towards sham values in HF-UN (ratio units: sham, $0.212 \pm 0.07$, $n = 53$; HF, $0.162 \pm 0.04$, $n = 20$; HF-UN, $0.238 \pm 0.05$, $n = 47$; sham vs. HF, $P = 0.01$, HF vs. HF-UN, $P = 0.001$; sham and HF-UN were not statistically different). HF cells showed a prolonged time to peak of the $Ca^{2+}$ transient as well as time to 50% and 90% decline in the $Ca^{2+}$ transient. These features were also normalized by HF-UN (*Figure 1*). The variance of the time-to-peak of the $Ca^{2+}$ transient measured at each
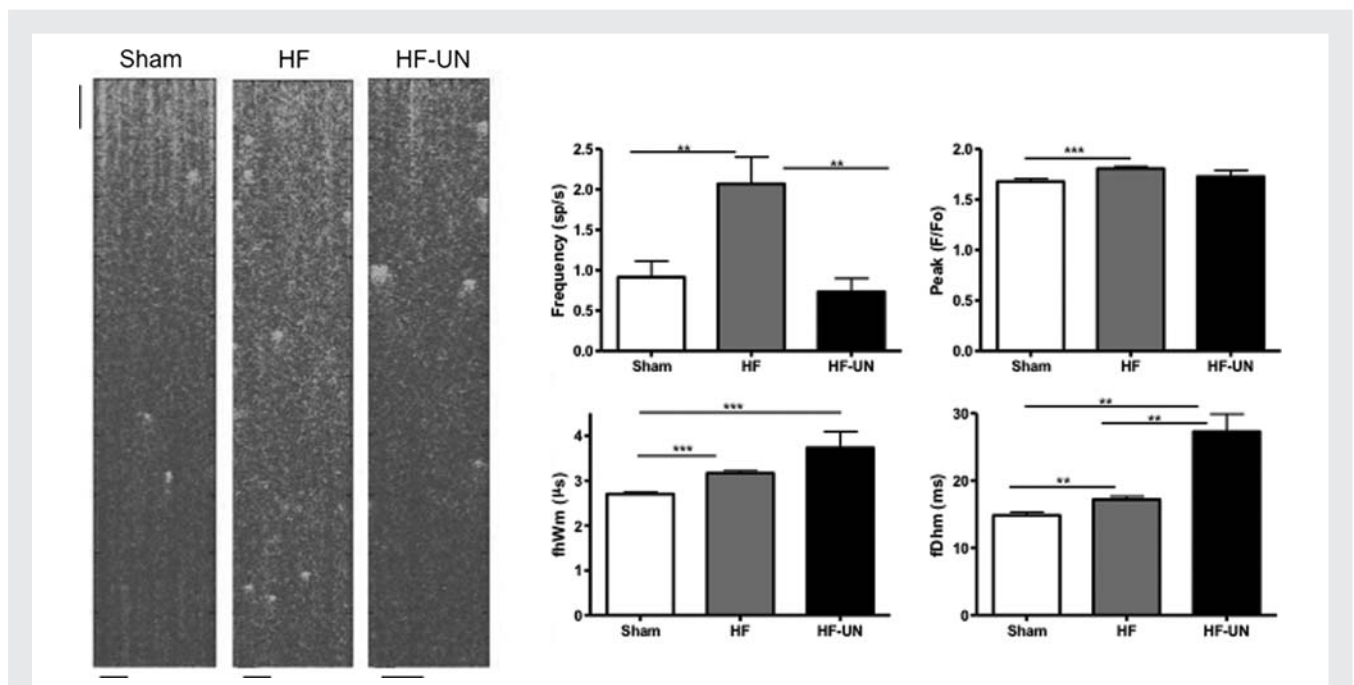


**Figure 2** Mechanical unloading (HF-UN) normalizes $Ca^{2+}$ spark frequency. Heart failure (HF) cells showed a higher $Ca^{2+}$ spark frequency, peak, width, and duration. Mechanical unloading normalized $Ca^{2+}$ spark frequency and increased width and duration. Mechanical unloading did not influence $Ca^{2+}$ spark peak. The vertical scale bar indicates 280 ms and the horizontal scale bar indicates 100 pixels (sham $n = 45$, HF $n = 34$, HF-UN $n = 36$).

pixel is taken as an index of CICR dyssynchrony and was increased in HF cells, but recovered in HF-UN (*Figure 1*). This suggests that the stimulus activates $Ca^{2+}$ release throughout the cell more uniformly in HF-UN. There are a number of possible causes for this normalization of CICR including normalized L-type $Ca^{2+}$ channel activity or normalized RyR function. Another possibility is that coupling between these trigger and release sites, which partly sets the gain of the positive feedback component of CICR, is altered.

## Raised $Ca^{2+}$ spark frequency is reduced by mechanical unloading

The spontaneous opening of RyR clusters can be characterized by measuring $Ca^{2+}$ sparks. $Ca^{2+}$ spark frequency was increased in HF cells compared with sham cells, but normalized after HF-UN (*Figure 2*). HF cells had a significantly higher $Ca^{2+}$ spark peak amplitude compared with sham myocytes, but unloading did not decrease $Ca^{2+}$ spark peak amplitude. $Ca^{2+}$ spark width and duration were increased by HF and mechanical unloading (*Figure 2*).
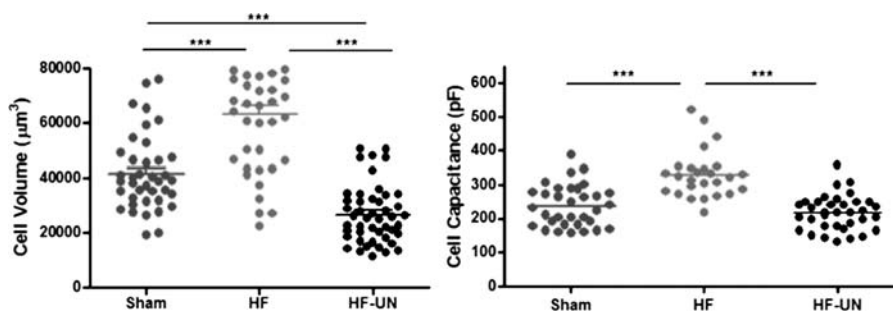


**Figure 3** Mechanical unloading (HF-UN) caused a regression of cellular hypertrophy. Mechanical unloading caused a regression of the cellular hypertrophy observed in heart failure (HF), to below sham levels. The cell capacitance, a measure of cell area, was increased in HF and reduced by mechanical unloading. For cell volume, sham $n = 39$, HF $n = 40$, HF-UN $n = 47$; for cell capacitance, sham $n = 33$, HF $n = 26$, HF-UN $n = 35$).
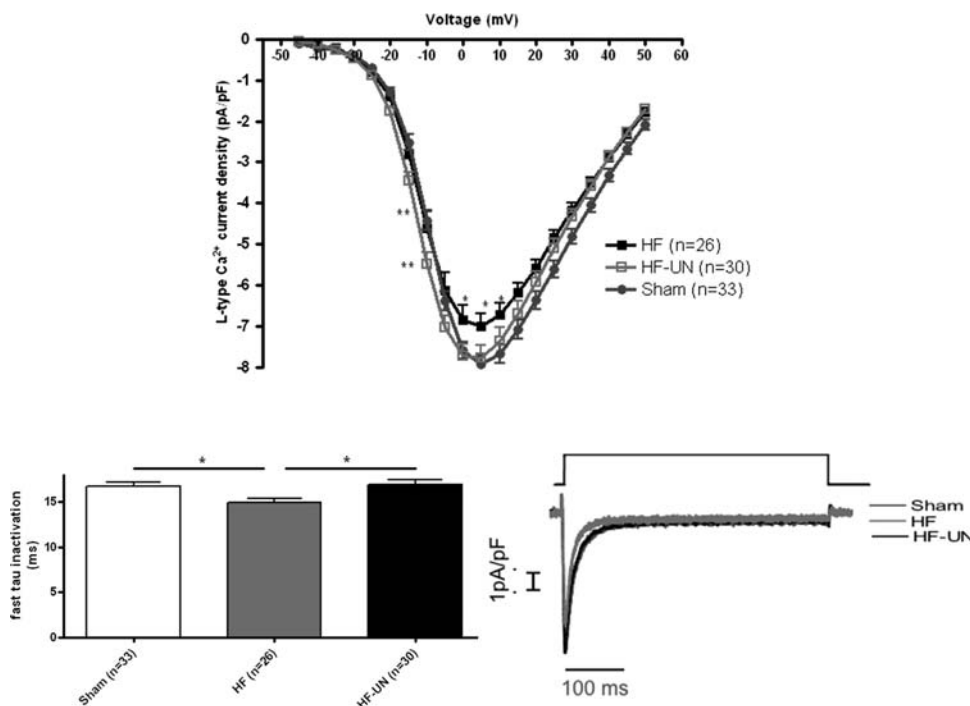


**Figure 4** Mechanical unloading (HF-UN) recovers the depressed L-type $Ca^{2+}$ channel density observed in heart failure (HF). Raw traces of L-type $Ca^{2+}$ current are shown. The L-type $Ca^{2+}$ current density was reduced in HF and normalized by mechanical unloading (sham $n = 33$, HF $n = 26$, HF-UN $n = 35$). Fast tau ($Ca^{2+}$-dependent) inactivation is shown in the bottom left.

## Mechanical unloading causes a regression of cellular hypertrophy

We assessed the volume of single cardiomyocytes using three-dimensional reconstruction of di-8-ANNEPPS images. Mechanical unloading induced a regression of hypertrophy, and average cell volume was smaller than for sham cells (*Figure 3*).

## Depressed L-type Ca$^{2+}$ channel activity is rescued by mechanical unloading

L-type Ca$^{2+}$ current activity was depressed in HF and was normalized in HF-UN (*Figure 4*). Cell capacitance, an index of cell surface area, showed a normalization to sham levels in HF-UN (*Figure 4*). The rate of activation was unaffected (data not shown), but the rate of fast, Ca$^{2+}$-dependent inactivation was faster in HF and was normalized in HF-UN (*Figure 4*).

## Mechanical unloading restores normal transverse tubule structure

Heart failure reduced the t-tubule density significantly compared with sham, and this was recovered by HF-UN (*Figure 5*). The deterioration in the regularity of the t-tubule system in HF, as measured by the power of the dominant frequency of the Fourier transform, was also normalized by HF-UN (*Figure 5*).

## Transverse tubule improvements after mechanical unloading are accompanied by improved cell surface structure

In sham cells, clearly defined z-grooves were present which contain the t-tubule openings. This was characterized by a high z-groove index. The cell surface was flattened and distorted in HF, with a reduction in the z-groove index. These features recovered after HF-UN (*Figure 6*).

## Mechanical unloading restores normal transverse tubule microarchitecture

Transverse tubule microarchitecture was assessed using transmission electron microscopy of single cardiomyocytes. The t-tubule lumens were reduced in density and dilated in HF. HF-UN normalized these parameters towards sham levels (*Figure 7*).
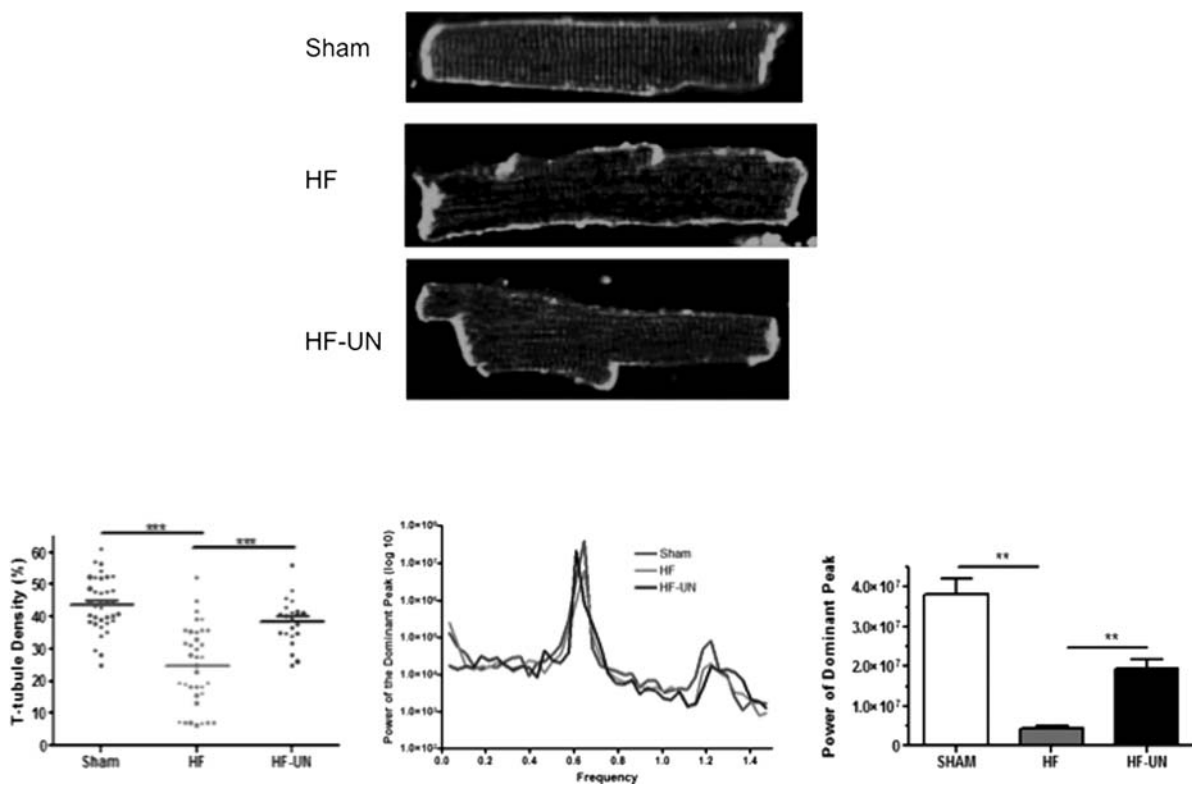


**Figure 5** Mechanical unloading (HF-UN) restores the normal transverse tubule (t-tubule) density and regularity. Single ventricular cardiomyocytes stained with di-8-ANEPPS are shown. Heart failure (HF) resulted in a reduced t-tubule density and a lower power of the dominant peak of the Fourier transform of the t-tubules (an index of t-tubular regularity). Mechanical unloading improved t-tubule density and regularity (sham $n = 40$, HF $n = 32$, HF-UN $n = 27$).
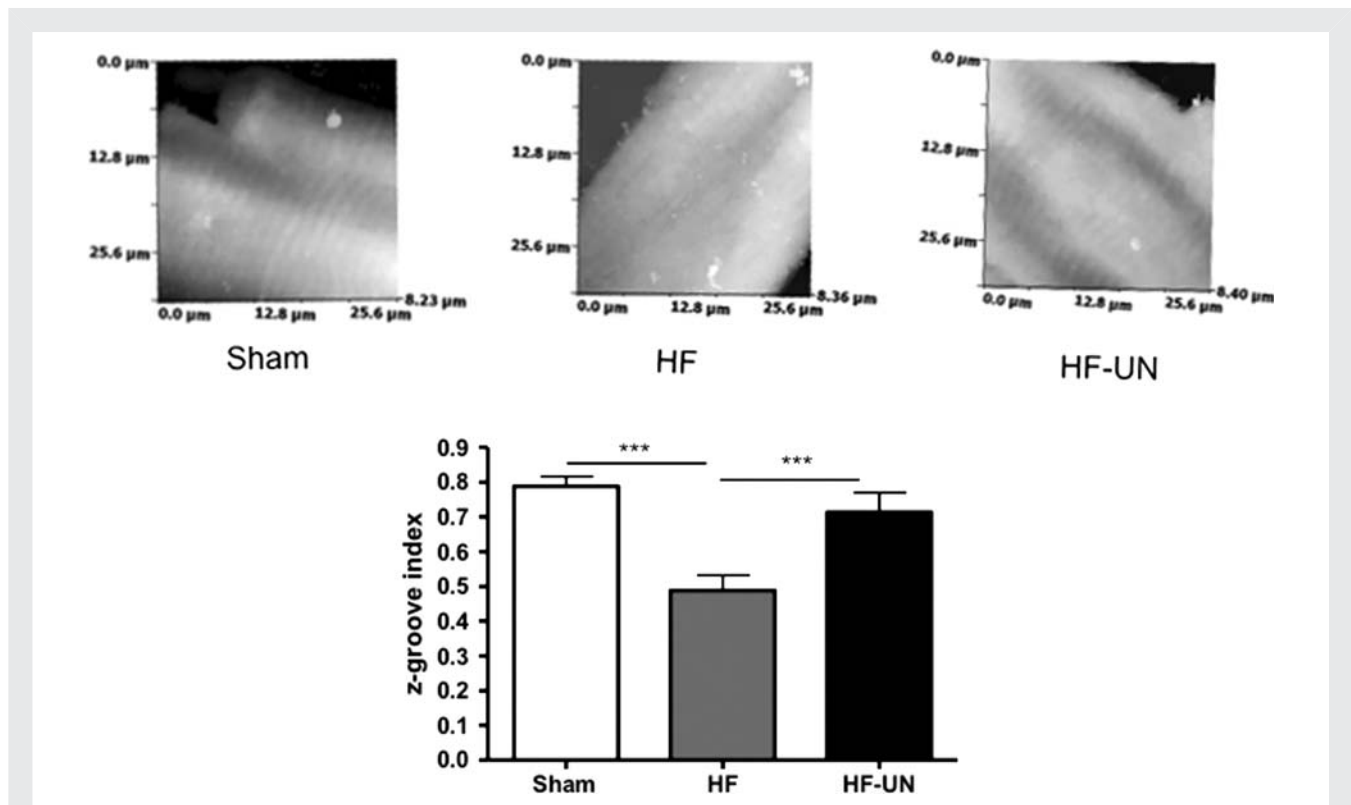
**Figure 6** Mechanical unloading (HF-UN) restores normal cell surface architecture. The z-groove index is an index of the regularity of the cell surface. This was reduced in heart failure (HF) and normalized by mechanical unloading (sham $n = 14$, HF $n = 25$, HF-UN $n = 17$).

## Mechanical unloading restores dihydropyridine receptor–ryanodine receptor coupling

Because the repair of the t-tubule system was associated with functional improvements to the CICR process, we assessed whether structural restoration of the DHPR–RyR relationship might account for the improved cellular $Ca^{2+}$ handling. In HF, the degree of co-localization of DHPRs and RyRs was reduced, but partially recovered in HF-UN (*Figure 8*).

## Junctophilin-2 is not directly responsible for improved dyadic coupling

Junctophilin-2 has been proposed as a regulator of the t-tubule system which is responsible for coupling t-tubule and SR membranes. Its expression is reduced with the progression from hypertrophy to HF,[8] possibly due to increasing overload. To test whether the improvements in t-tubule structure were due to changes in JP-2 expression, we performed western blotting (Supplementary material online, *Figure S1*). JP-2 expression was significantly reduced in HF, but not recovered by HF-UN.
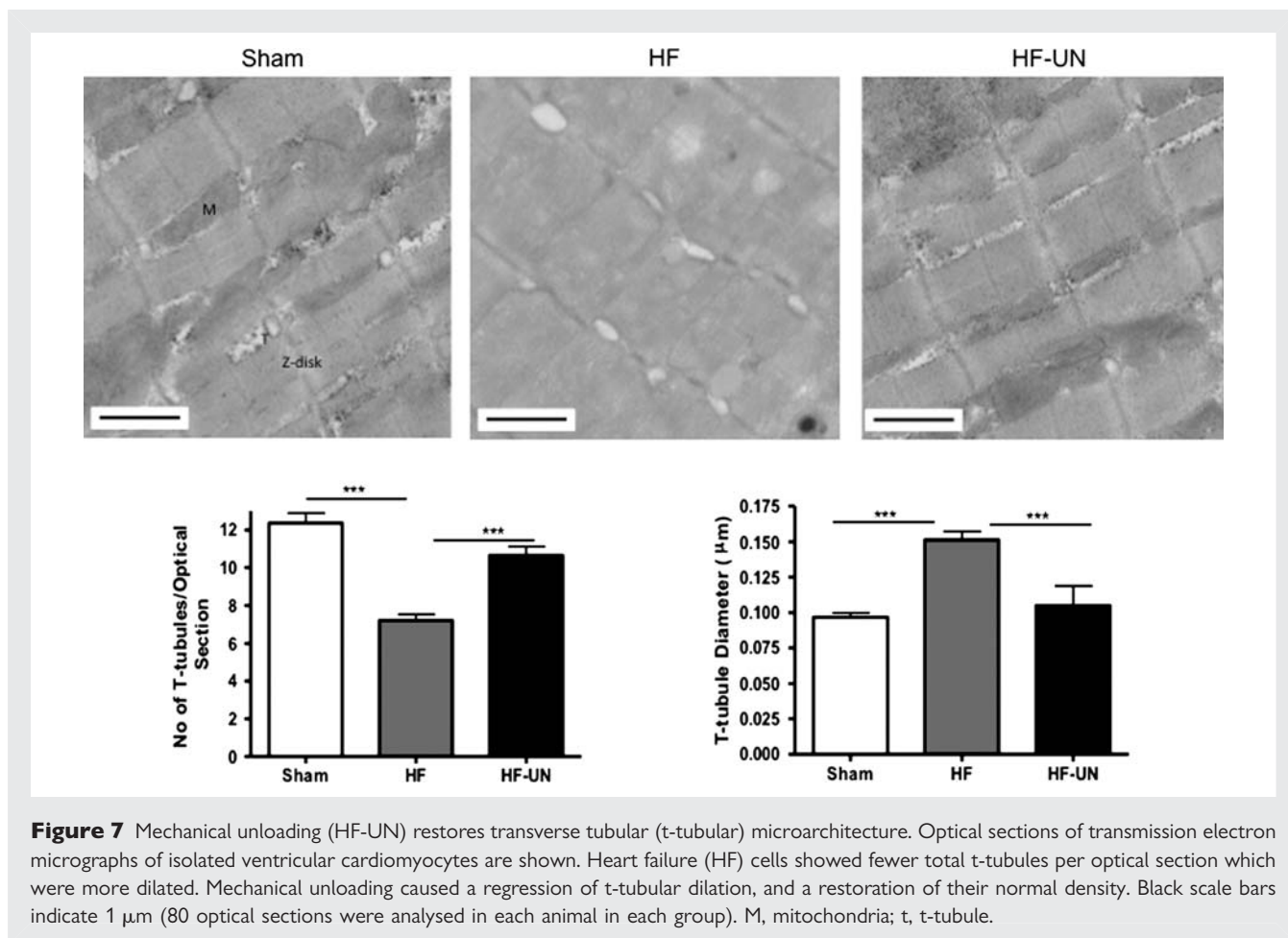
## Discussion

We report that mechanical unloading of failing hearts results in regression of a number of pathological $Ca^{2+}$ handling properties. Unloading normalized the t-tubule density and regularity, which

resulted inDHPR–RyR recoupling. Unloading also normalized the L-type $Ca^{2+}$ current density.

## $Ca^{2+}$ handling improvements

Heart failure cardiomyocytes show disrupted $Ca^{2+}$ release synchronicity.[2,3,5–7,24,25] Our study also shows $Ca^{2+}$ transients severely disrupted in HF and with multiple points of delayed SR $Ca^{2+}$ release across the cell compared with sham. Points of delayed SR $Ca^{2+}$ release are localized to gaps in the t-tubule system.[3,6,7] SR $Ca^{2+}$ release is initiated at t-tubules but propagates more slowly to activate RyRs in regions devoid of t-tubules.[2] Our immunofluorescence experiments suggest that the irregular nature of the t-tubule system may account for a considerable element of $Ca^{2+}$ transient disruption. The reappearance of regular t-tubules probably accounts for the improvements in $Ca^{2+}$ release synchronicity by improving DHPR–RyR coupling.

We also report SR $Ca^{2+}$ content depression in HF, a critical determinant of $Ca^{2+}$ transient amplitude. The SR $Ca^{2+}$ content improves after mechanical unloading, which may contribute to the improvement in the $Ca^{2+}$ transient amplitude. Changes to the RyR phosphorylation status and action potential (AP) morphology are features which we did not investigate that may contribute to the changes in the $Ca^{2+}$ transient. Previous studies suggest that mechanical unloading alone does not influence the AP morphology,[10] but whether this is true in the context of the failing heart is not known. Recent computational studies[26] suggest that AP changes

**Figure 7** Mechanical unloading (HF-UN) restores transverse tubular (t-tubular) microarchitecture. Optical sections of transmission electron micrographs of isolated ventricular cardiomyocytes are shown. Heart failure (HF) cells showed fewer total t-tubules per optical section which were more dilated. Mechanical unloading caused a regression of t-tubular dilation, and a restoration of their normal density. Black scale bars indicate 1 μm (80 optical sections were analysed in each animal in each group). M, mitochondria; t, t-tubule.

*per se* are not primarily responsible for dyssynchronous $Ca^{2+}$ release in rodent HF, and that other changes, including t-tubule abnormalities, may be more important. In larger species, such as man, AP changes are an important contributor to dyssynchrony.

$Ca^{2+}$ sparks are due to opening of RyR clusters, and their dynamics are a reflection of the status of these clusters. In HF, spontaneous $Ca^{2+}$ sparks occur more frequently at gaps in the t-tubules, suggesting that they were generated by uncoupled RyRs.[27] This uncoupling is caused by disruptions to the t-tubule system[3,28] and could explain our finding that global $Ca^{2+}$ spark frequency is raised in HF. This may also explain why $Ca^{2+}$ spark frequency is normalized after the reappearance of t-tubules in HF-UN. The $Ca^{2+}$ spark amplitude, duration, and width are determined by mechanisms which are incompletely understood, but include the SR $Ca^{2+}$ content, the functional status of RyRs, the buffering properties of the cells, and possibly the activity of the $Na^+$/$Ca^{2+}$ exchanger (NCX).[29–31] All these elements are affected in HF and their interaction sets the $Ca^{2+}$ spark amplitude (and other $Ca^{2+}$ spark features) which is increased in our model, despite reduced SR $Ca^{2+}$ content. Direct effects of phosphorylation on RyR open probability, due to involvement of $Ca^{2+}$/calmodulin-dependent protein kinase II[32] or accessory proteins[33] in HF, may occur, but whether these aspects contribute to the $Ca^{2+}$ spark changes observed here is not known. The increase in the duration
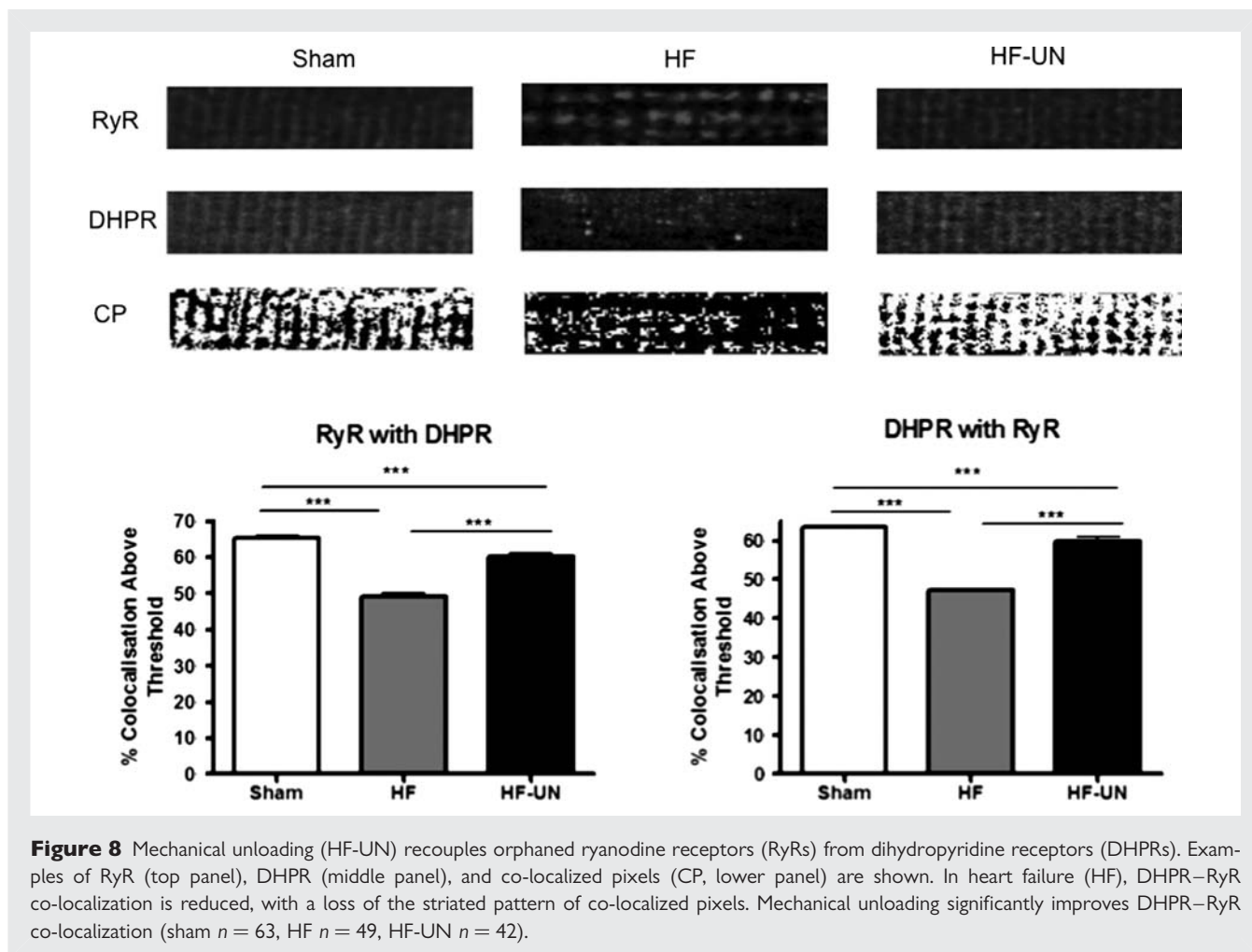
of the $Ca^{2+}$ sparks observed in HF and HF-UN may be due to a number of factors, including the phosphorylation status of RyR clusters and the interaction between RyR clusters and other ion channels including NCXs. Recent work shows that gaps in the t-tubule system may result in displacement of local NCXs away from RyRs,[48] which can prolong the $Ca^{2+}$ spark as NCX is not available to terminate the local increase in $[Ca^{2+}]_i$.

## Regression of cellular hypertrophy

Mechanical unloading induces a regression of cellular hypertrophy.[10,34] Mechanical unloading of failing hearts does not cause as large a reduction in cell size as for unloading of normal hearts. Although profound reductions in cell volume, atrophy, may induce dysfunction, there is no direct relationship between cell size and CICR efficacy.[10,19,35,36]

## L-type $Ca^{2+}$ current changes

We have previously reported that L-type $Ca^{2+}$ current density is reduced in HF,[20] possibly due to reduced numbers of L-type $Ca^{2+}$ channels, following the loss of t-tubule membrane. Other studies do not show a depressed L-type $Ca^{2+}$ current density in animal models of HF.[37,38] L-type $Ca^{2+}$ current depends on both the number and the activity of L-type $Ca^{2+}$ channels. Since there is a reduction in the t-tubule density in our model, it is likely

**Figure 8** Mechanical unloading (HF-UN) recouples orphaned ryanodine receptors (RyRs) from dihydropyridine receptors (DHPRs). Examples of RyR (top panel), DHPR (middle panel), and co-localized pixels (CP, lower panel) are shown. In heart failure (HF), DHPR–RyR co-localization is reduced, with a loss of the striated pattern of co-localized pixels. Mechanical unloading significantly improves DHPR–RyR co-localization (sham $n = 63$, HF $n = 49$, HF-UN $n = 42$).

that the number of channels is reduced. Unchanged L-type $Ca^{2+}$ current density in HF models may be due to increased single channel activity (but reduced numbers).[39–42] We found normalization of L-type $Ca^{2+}$ channel activity with unloading, which is due either to new t-tubules containing L-type $Ca^{2+}$ channels or to increased conductance amongst existing channels. In this study, we report a quicker rate of fast inactivation ($Ca^{2+}$ dependent) of the L-type $Ca^{2+}$ current in HF. We[49] and several other groups have previously reported that fast tau is increased in HF. It is not clear what the mechanism of this surprising result is, whether this is the result of direct alterations of the channel, its relationship with accessory proteins, or its regulation by external factors.

## Transverse tubule physiology

Our study confirms deterioration in t-tubule structure in HF models[2–4,43] and in diseased human myocardium.[5] We report for the first time recovery of the t-tubule system in post-ischaemic cardiomyopathy by reducing mechanical load. Only one study, using exercise in murine diabetic cardiomyopathy, has previously shown that t-tubule recovery is possible.[44] We show that unloading recovers the t-tubule system and normalizes CICR by DHPR–RyR recoupling. Although we cannot claim a causal relationship, this finding strengthens the argument that

the t-tubule dysfunction of the failing heart is an important factor in the derangement of local CICR in HF. One limitation of this study is that we did not measure the protein expression of DHPRs and RyRs. Further studies should do so, as this would clarify the mechanism of reduced L-type $Ca^{2+}$ current. It would also clarify whether replenishment of RyRs as well as reorganization of RyRs is involved in the cellular recovery observed in HF. The z-groove index provides a measure of the regularity of the membrane structure, which may or may not impact on the DHPR–RyR coupling. In all conditions studied so far, changes to the z-groove index are associated with dysfunctional CICR. An important question for future studies is to elucidate the relationship between the z-groove, subcellular structures, and the t-tubule membrane.

Our model of HF is driven by myocardial overload as it involves a reduction in cell number but leaves the remainder of the heart normally perfused. Myocardial overload results in t-tubular disarray and impairment of local CICR.[8] Conversely, prolonged mechanical unloading of normal hearts results in a reduction in the regularity of the t-tubule system and also impairs CICR.[10] We proposed that the t-tubule system is dependent on the chronic load of the myocardium, with physiological levels of load associated with an 'optimal' t-tubule structure which

promotes efficient CICR.[9] Prolonged overloading or unloading results in changes to the t-tubule system which are detrimental to CICR. Here we show that normalizing the load of a failing heart can induce reverse remodelling of the t-tubule system and normalize many aspects of cellular $Ca^{2+}$ handling. Whether further unloading would result in detrimental cellular remodelling is uncertain.

## Molecular mechanisms underlying the recovery of transverse tubule structure

The mechanisms mediating the load dependence of the t-tubule structure are poorly defined, but a number of molecules may be involved. Such molecules include Telethonin, which has recently been shown to be both stretch sensitive and involved in orderly t-tubule formation.[45] The physiological regulators of other proteins, such as BIN1 which is involved in t-tubule biogenesis and the shuttling of L-type $Ca^{2+}$ channels to the membrane, is not known.[46,47] JP-2, a protein thought to promote dyad coupling, is reduced as cardiac hypertrophy develops into HF,[8] and may therefore be load sensitive. JP-2 knockdown disrupts t-tubule structure and results in HF.[23] In this study, we confirm the finding that JP-2 is depressed in HF. However, we find no increase in JP-2 after mechanical unloading when t-tubule structure is improved. There are a number of possible explanations for this. First, JP-2 may not be required in abundance in a mechanically unloaded heart, where low levels of JP-2 may be sufficient to support the t-tubule network in these small cells. Secondly, there may be other proteins of unknown identity which play a role. Thirdly, the phosphorylation status and/or conformational state of these proteins may be altered in HF and mechanical unloading, which could be responsible for the changes observed. How these molecular pathways interact in health and disease is a central question for future studies.

## Non-recovered features

Mechanical unloading did not recover all of the parameters investigated. Although the important feature of $Ca^{2+}$ spark frequency was normalized, the width and duration of the $Ca^{2+}$ sparks were deranged further with respect to sham. It is not clear what mechanism drives this, but it may include changes to the phosphorylation status of the RyR clusters which could be altered by unloading. Cell volume regressed profoundly to below sham levels, indicating the large diminution of cell size which accompanies mechanical unloading.

This study reflects many previous reports of mechanical unloading-induced regression of hypertrophy, and for the first time documents changes to the cell membrane structure responsible for effective excitation–contraction coupling as an important player in this process.

## Conclusions

In conclusion, we show that mechanical unloading of failing hearts produces recovery of the t-tubule structure with improvement of local CICR, probably as a result of enhanced DHPR–RyR coupling. These changes constitute a possible cellular mechanism for the initial improvements in cardiac function seen after LVAD therapy, although this requires direct testing in future studies. The mechanisms mediating the retubulation observed in this study require further investigation and may highlight potential new therapeutic targets.

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