



Membrane remodelling triggers maturation of excitation–contraction coupling in 3D-shaped human-induced pluripotent stem cell-derived cardiomyocytes

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Abstract

The prospective use of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) for cardiac regenerative medicine strongly depends on the electro-mechanical properties of these cells, especially regarding the Ca²⁺-dependent excitation–contraction (EC) coupling mechanism. Currently, the immature structural and functional features of hiPSC-CM limit the progression towards clinical applications. Here, we show that a specific microarchitecture is essential for functional maturation of hiPSC-CM. Structural remodelling towards a cuboid cell shape and induction of BIN1, a facilitator of membrane invaginations, lead to transverse (t)-tubule-like structures. This transformation brings two Ca²⁺ channels critical for EC coupling in close proximity, the L-type Ca²⁺ channel at the sarcolemma and the ryanodine receptor at the sarcoplasmic reticulum. Consequently, the Ca²⁺-dependent functional interaction of these channels becomes more efficient, leading to improved spatio-temporal synchronisation of Ca²⁺ transients and higher EC coupling gain. Thus, functional maturation of hiPSC-cardiomyocytes by optimised cell microarchitecture needs to be considered for future cardiac regenerative approaches.

Keywords hiPSC cardiomyocytes · 3D reshaping · t-tubules · BIN1 · Maturation · Excitation–contraction coupling

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Introduction

The technology to generate cardiomyocytes from human-induced pluripotent stem cells (hiPSC-CM) represents a great breakthrough for pharmacological tests and disease modelling in cardiovascular research [12, 22] and fuels new hope for cell-based therapy of patients with heart failure [28, 42]. Human iPSC-CM reveal all essential cardiac features at the electro-mechanical level but retain a premature phenotype compared to ventricular cardiomyocytes of the adult heart. In particular, cell morphology and subcellular microarchitecture remain underdeveloped, lacking the typical anisotropic cuboid shape of adult ventricular cardiomyocytes and the establishment of transverse-axial (t)-tubular network (aka TATS) of the sarcolemma [29, 46]. As a consequence, myofibrils are distributed in a diffuse manner across the rather polygonal cell shape leading to underdeveloped sarcomeres and inefficient contraction [33, 58]. Moreover, the absence of t-tubules, which provide the structural basis for functional interaction between the sarcolemmal L-type Ca²⁺ channel (LTCC) and the intracellular Ca²⁺ release channel, the type 2 ryanodine receptor (RyR2),

located at the sarcoplasmic reticulum (SR), is the reason for inefficient Ca^{2+} handling in hiPSC-CM [2, 7]. In adult cardiomyocytes, efficient functional coupling between LTCC and RyR2 is ensured by close proximity of both channels of about 20 nm, a distance that is provided by the dyadic cleft between the t-tubular membrane and the SR [15, 49, 50]. Depolarization-induced activation of LTCCs leads to Ca^{2+} influx at the t-tubular membrane, which triggers further Ca^{2+} release by RyR2s needed for contraction, a process called Ca^{2+} -induced Ca^{2+} release (CICR) [5]. The efficiency of this excitation–contraction (EC) coupling mechanism is fundamentally dependent on the amplification of the initial Ca^{2+} influx by RyR2-mediated Ca^{2+} release and on the spatio-temporal synchronisation of this Ca^{2+} release throughout the cell, which is normally ensured by the well-organised t-tubular network deep into the cell [4, 9]. Absence or disruption of the t-tubular network may alter CICR leading to a reduction of the EC coupling gain, as known from cardiomyocytes derived from hypertrophied or failing heart [47, 57]. In hiPSC-CM, such a well-structured tubular network and consequently the formation of dyads are not yet developed causing abnormal and inefficient Ca^{2+} handling and EC coupling [61]. So far, the triggers for the development of t-tubules in premature cardiomyocytes have not yet been identified, and many approaches have been tested to drive these novel cardiomyocytes to further structural and functional maturation in vitro. They comprise biophysical and biochemical stimulations [36, 45, 59], co-cultures with cardiac non-cardiomyocytes [8, 19], cell or substrate patterning and tissue engineering [27, 40, 43, 46, 51].

Recent research has focussed intensively on the investigation of the effect of topographical and physical cues on the maturation of hiPSC-CM during culture. Cardiomyocytes grown on micropatterned substrates enhanced cell alignment and improved Ca^{2+} handling and contraction [23, 35, 40, 54]. In a recent study, we reported that single mouse iPSC-CM seeded into cuboid 3D micro-scaffolds produced strong tubular invaginations and revealed more robust Ca^{2+} signalling [46].

Apart from environmental parameters, the question arises which cellular mechanisms may be involved in tubulogenesis. Few proteins have come into the focus of attention as potential key players in the process of t-tubule and dyad formation, specifically caveolin-3, nexilin and junctophilin. While overexpression of caveolin-3 prevented the loss of tubular network in cardiomyocytes from pressure overload-induced failing mouse hearts [24], loss of nexilin, a component of cardiac dyads, resulted in a loss of the tubular network and disruption of dyads in ventricular cardiomyocytes [30]. Junctophilin-2 expression may be involved in t-tubule development and dyad stabilisation in hiPSC-CM [10, 37].

Here, we focus on the potential role of the bridging integrator-1 protein (BIN1) in t-tubule biogenesis [3, 14, 17].

As a member of the BAR (BIN1-amphiphysin/Rvs) domain superfamily, BIN1 is involved in multiple cellular processes including membrane trafficking, recycling and remodeling, cytoskeleton regulation, muscle development, DNA repair and apoptosis [39]. BIN1 has been identified in the brain, heart and skeletal muscle, and is composed of: (i) an amino-terminal BAR domain, which oligomerizes and forms a “banana-shaped” molecule to interact with plasma membrane lipids; (ii) a tissue-specific proline-rich middle linkage domain; and (iii) a carboxy-terminal SH3 domain interacting with intracellular proteins [15]. BIN1 has not only been shown to be indispensable for muscle development, t-tubule formation, Ca^{2+} homeostasis and fibre organisation of skeletal muscle cells [38, 52], but a reduction of BIN1 has also been described in diseased cardiomyocytes, thereby correlating with a disruption of the t-tubular network [18, 55].

To induce further maturation of hiPSC-CM, we merged two approaches and combined 3D reshaping together with BIN1 expression to trigger structural remodelling and maturation. We tested the hypothesis that reshaping hiPSC-CM in pre-designed cuboid 3D micro-scaffolds and overexpression of BIN1 lead to tubulogenesis and improve Ca^{2+} handling at the level of EC coupling. Our data demonstrate that a cardiomyocyte-specific microarchitecture comprising a t-tubular-like membrane network is required for efficient EC coupling in hiPSC-CM and, thus, for the development of robust and mature cardiomyocytes. Our findings open new avenues for future hiPSC-cardiomyocytes development and applications as cardiac cell grafts.

Methods

Human iPS cells and cardiogenic differentiation

Human iPS cell lines were kindly provided by Dr. Lukas Cyganek, Stem Cell Unit Göttingen, University Medical Center Göttingen (UMG). Wild-type iPSC line UMGi014-C clone 14 (isWT1.14) was generated from dermal fibroblasts using the integration-free Sendai virus and described previously [44]. Two other cell lines were used to replicate the representative experiments (ethical approval number for all hiPSC lines used in this study: S-455/2018). hiPSC were differentiated into cardiomyocytes by modulation of the Wnt/ β -catenin signalling pathway at 95–100% confluency as previously described [62]. After differentiation, cells were purified by metabolic selection using glucose-free and sodium–lactate-supplemented medium to prevent growth of non-cardiomyocytes. Finally, cells were maintained in RPMI plus B27 with insulin until further experiments. A detailed description of the differentiation and purification methods is provided in the Supplemental files.

General methods

Detailed methods for cardiomyocyte preparation from hiPSC and culture, 3D micro-scaffold production, SEM imaging, AAV6-mediated transduction, immunocytochemistry, PLA, live Ca²⁺ imaging, cellular electrophysiology, quantitative PCR, Western blotting and statistical analysis are provided in the Supplemental file section.

Results

BIN1 is successfully expressed in transduced hiPSC-CM

To test our hypothesis whether 3D reshaping induced structural remodelling, single hiPSC-CM were plated on a planar surface and in 3D micro-scaffolds with hexagonal and cuboid shapes. To recapitulate the rectangular structure of adult cardiomyocytes, 3D-cuboid scaffolds were designed accordingly to provide a long axis and spatial restrictions to further grow in height as described before [46]. Hexagonal 3D scaffolds with similar surface area were employed for direct comparison of cells growing in 3D-confined structures without cell elongation [46] (Fig. 1A, B). Adherent cells were transduced with an Adeno-associated virus 6 (AAV6) vector carrying BIN1 and a fluorescent marker (dsRed), or a control vector containing only dsRed. After 5–6 days of transduction, cells were utilised for further experiments. As shown in Fig. 1C, hiPSC-CM revealed strong levels of red fluorescence confirming successful transduction. BIN1 expression was significantly boosted at both mRNA ($p=0.005$) and protein ($p=0.017$) levels in hiPSC-CM transduced with BIN1 compared to control, which only showed low levels of BIN1 protein (Fig. 1D, E). We also assessed the effect of BIN1 overexpression on the mRNA expression levels of proteins of the contractile apparatus, including myofilaments like cardiac troponin T (cTNT), myosin heavy chain 6 (MYH6), MYH7 and α -actinin (ACTN2), and Ca²⁺-handling proteins such as the L-type Ca²⁺ channel (CACNA1C), RYR2, SERCA2 (ATP2A2) and Na⁺/Ca²⁺ exchanger (NCX). The mRNA levels of these targets remained unaffected by BIN1 overexpression.

3D reshaping and BIN1 overexpression lead to adaptations in cell morphology and microarchitecture

In comparison to rod-shaped adult ventricular cardiomyocytes, which present parallel-aligned and organised myofibrils, 2D-cultured hiPSC-CM are rather flat with no defined long axis formation. To assess the shape of hiPSC-CM and myofibril structures in 3D micro-scaffolds, the organisation of the

sarcomeric α -actinin and actin was investigated. As shown in Fig. 2A, non-patterned hiPSC-CM revealed a round shape with irregular and random orientation of actin filaments throughout the cell. The distribution pattern of myofilaments in hexagonally shaped hiPSC-CM was also similar to non-patterned cells. Conversely, hiPSC-CM grown in cuboid micro-scaffolds displayed an elongated anisotropic shape with well-organised myofibrils and parallel-aligned α -actinin, indicating sarcomeric units. This remarkable alignment of myofibrils along the long axis of the cell in a cuboid scaffold demonstrates the great power of reshaping to induce subcellular remodelling processes in hiPSC-CM.

In the next step, we investigated the synergistic potency of 3D reshaping and BIN1 overexpression on triggering the generation of membrane invaginations forming t-tubules, which are critical for proper EC coupling. Immunolabeling of BIN1 confirmed the expression of BIN1 in transduced hiPSC-CM presenting strong sarcolemmal invaginations indicative of the development of an early but still unstructured tubular network (Fig. 2B, Figures SIA, B). In comparison, hiPSC-CM transduced with the control vector did not reveal any substantial levels of BIN1 expression nor plasma membrane invaginations (Figure SIC). For further detailed investigations of the origin of the tubular network, cells were recorded using the fluorescent membrane dye di-8-ANEPPS in confocal live imaging experiments. Since this dye only incorporates within the plasma membrane, but not in subcellular membrane compartments of intact cells, fluorescence signals of tubular structures derived exclusively from plasma membrane invaginations. The confocal images showed extensive tubular network generation in 3D-reshaped BIN1-overexpressing hiPSC-CM. Moreover, statistical analysis confirmed that there was a significant dual effect of 3D reshaping and BIN1 overexpression on increasing tubule density as assessed by two-way ANOVA ($F(2, 125)=20.034, p<0.05$; Fig. 2C, D). Interestingly, 3D reshaping without BIN1 overexpression also elicited remarkable tubule generation compared to control (non-patterned cells), but with high variability (Fig. 2D). Adding BIN1 to it significantly enhanced t-tubule formation and thereby reduced this large variability in all groups. So, the results demonstrate a combined effect of reshaping and BIN1 expression with regard to t-tubule density. We further confirmed that both 3D reshaping and BIN1 overexpression significantly induced tubular membrane network in hiPSC-CM derived from another stem cell line with a different genetic background (Figure SII).

Membrane remodelling reorganises the expression pattern of Ca²⁺ handling proteins and induces dyad formation

Since the close proximity of LTCCs and RYR2s is vital for efficient CICR and EC coupling, in the next step, we investigated the influence of 3D reshaping and BIN1-induced

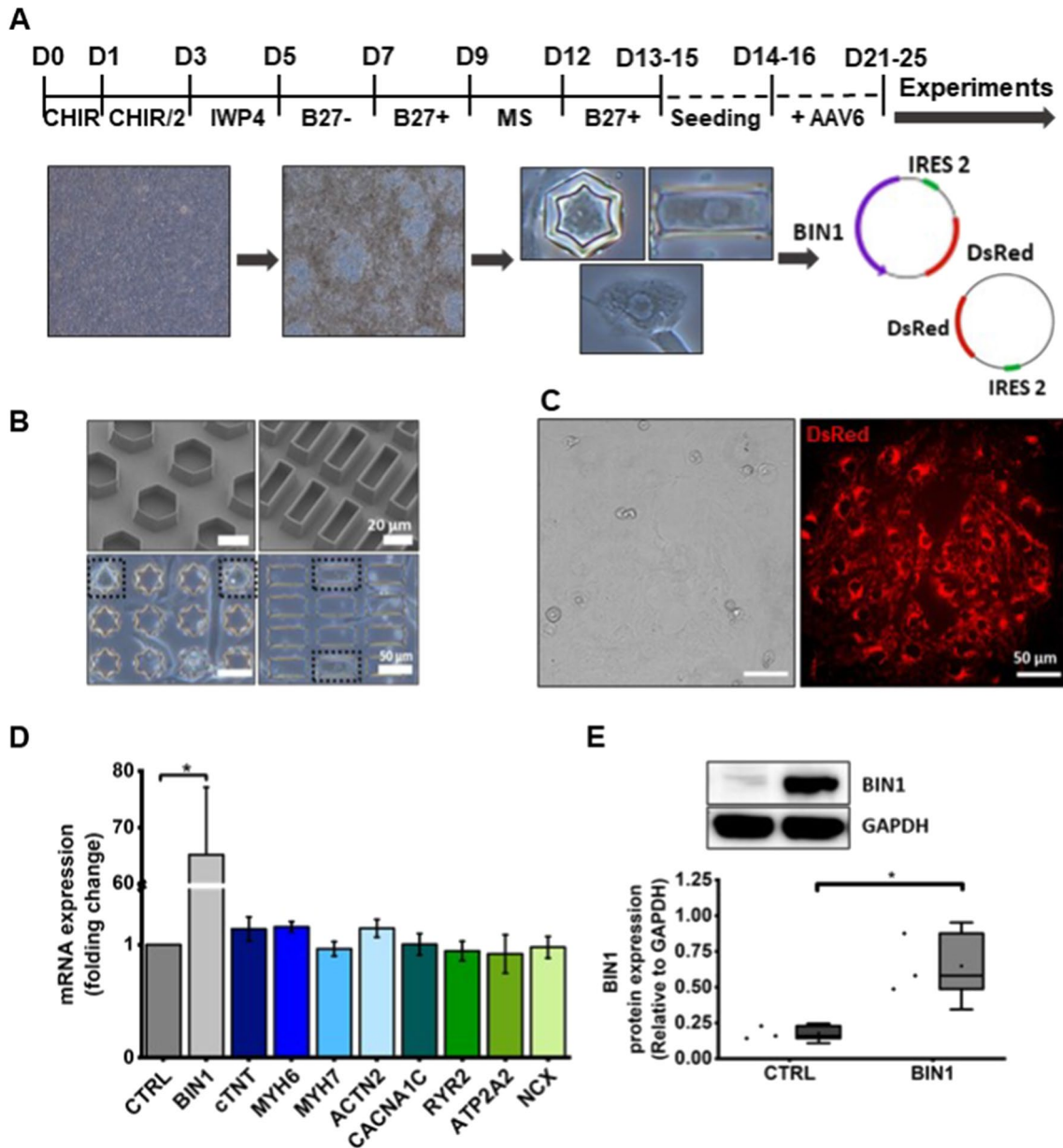


Fig. 1 BIN1 is successfully expressed in transduced hiPSC-CM. **A** Schematic timeline of cardiogenic differentiation and subsequent experimental design. **B** 3D scaffolds in hexagonal (left images) and cuboid (right images) shapes. **C** Representative images of bright field (left image) and dsRed expression (right image) of hiPSC-CM. **D** Quantification of mRNA expression levels relative to control, meas-

ured by real-time qRT-PCR ($N=4-5$, $*p<0.05$ for BIN1 vs. CTRL). The list of primers can be found in Table S1. **E** Immunoblot of BIN1 protein expression in transduced hiPSC-CM. Quantification of the change in protein abundance of BIN1 ($N=3$, $*p<0.05$). Data are presented as mean \pm SE (bar graph) or mean \pm SD (box blot), significance tested by Student's t test

membrane invaginations on the expression pattern of these Ca^{2+} channels. Immunolabeling of BIN1 and LTCC illustrated that BIN1 enhanced LTCC clustering along membrane tubules (Fig. 3A). This notion is further supported by the overlapping fluorescence signal peaks in the merged line profiles (right panel). Moreover, double staining of RYR2s and LTCCs manifested spatial clustering of these proteins relative to each other. The line profiles of both Ca^{2+} channels

showed a higher degree of fluorescent signal overlap in cuboid BIN1-overexpressing hiPSC-CM as compared to control non-patterned and hexagonally shaped cells (Fig. 3B, Figure SIIIA). In line with this experiment, we performed highly specific and sensitive proximity ligation assays (PLA) to further verify the close localization of LTCCs and RYR2s within the BIN1-induced vicinity of t-tubules and SR. Both 3D reshaping and BIN1 overexpression had a significant

effect on the density of closely located LTCCs and RyR2s in hiPSC-CM; however, statistically seen, there was no interaction between all shapes as assessed by two-way ANOVA ($F(2, 139) = 2.598, p = 0.078$; Fig. 3C, D, Figure SIII B). Detailed analysis showed that PLA signal density was significantly higher in cuboid, BIN1-expressing hiPSC-CM compared to non-patterned BIN1-expressing and cuboid control hiPSC-CM. This suggests that BIN1 expression together with rectangular reshaping of hiPSC-CM may have an additive effect on augmenting the probability of forming dyadic structures. Overall, these results support the concept that BIN1 may serve as a local anchor for stabilisation of LTCCs and RYR2s in close proximity in t-tubular and SR membranes to trigger the formation of functional dyads in hiPSC-CM, promoting co-localization and further functional interaction.

Characterisation of I_{CaL} properties in reshaped BIN1-overexpressing hiPSC-CM

The spatial rearrangement of LTCCs and RyR2s fuels the assumption that enhanced dyad formation may have functional consequences for the EC coupling mechanism. To investigate Ca^{2+} handling in reshaped and BIN1-overexpressing cells, we first characterised Ca^{2+} influx via LTCCs in more detail. Single hiPSC-CM were patch clamped in the whole-cell configuration and membrane Ca^{2+} currents (I_{CaL}) were measured using the indicated voltage clamp protocols. In each cell, the current–voltage (IV) relationship, the voltage dependence of activation and the voltage dependence of inactivation were examined with independent voltage protocols. From the IV curve, the current amplitudes were measured to assess the peak Ca^{2+} current amplitudes in dependence of the clamped membrane potential. Total membrane current was then normalised to cell capacitance to obtain the LTCC current density per cell, a measure to compare the different values of different cells and cell sizes. Figure 4A illustrates the IV curves of I_{CaL} recorded from hiPSC-CM of the different experimental groups. On average, the peak I_{CaL} amplitudes at different potentials were not significantly different in the experimental groups. This finding was further confirmed by measuring and comparing the voltage-dependent activation and inactivation states of I_{CaL} . From the steady-state activation and inactivation curves (Fig. 4D–F), the half-maximal voltages for activation and inactivation were derived (Fig. 4G and H). Both values are required from each cell to characterise the LTCC gating kinetics. As expected, our data demonstrate that $V_{1/2}$ of I_{CaL} activation and inactivation of the different experimental groups were not statistically different from each other. To investigate the fast Ca^{2+} -dependent inactivation of I_{CaL} , the first time constant (τ_1) of current decay measured

at +10 mV was compared among the different experimental groups (Figs. 4F, G). Again, no significant differences were detected.

Characterisation of Ca^{2+} spark events in reshaped BIN1-overexpressing hiPSC-CM

Since I_{CaL} properties were unchanged in the different experimental groups, we took a closer look at RyR2 function. To this end, we measured spontaneous Ca^{2+} release events and characterised the spatial and temporal properties of Ca^{2+} sparks. Spontaneous Ca^{2+} sparks were recorded in the confocal line-scan mode using the Ca^{2+} -sensitive dye fluo-4 (Fig. 5A). Interestingly, 3D reshaping of hiPSC-CM resulted in a significant reduction of spark frequency (Fig. 5B) and spark duration (Fig. 5C). In addition, BIN1 overexpression significantly reduced spark width (Fig. 5D). Two-way ANOVA revealed a synergistic effect of 3D reshaping and BIN1 overexpression on the temporal (FDHM: $F(2, 132) = 3.534, p = 0.032$) and spatial dynamics (FWHM: $F(2, 131) = 3.401, p = 0.036$) of the Ca^{2+} sparks.

Maturation of Ca^{2+} transient dynamics in 3D-reshaped BIN1-overexpressing hiPSC-CM

Having shown that 3D reshaping and BIN1 overexpression elicit structural remodelling, we, in turn, tested our hypothesis that structural remodelling also favours functional adaptations. To examine this concept, we recorded spontaneous Ca^{2+} transients in confocal line-scan imaging using the Ca^{2+} -sensitive fluorescent indicator fluo-4. Representative line-scan images and line profiles of each experimental group are summarised in Fig. 6A. Detailed analysis of Ca^{2+} transient kinetics revealed that 3D reshaping significantly reduces time-to-peak (TTP, $p < 0.001$, Fig. 6B) and decay times ($p < 0.001$, Fig. 6C), while BIN1 overexpression and 3D reshaping significantly interact to decrease the time of full duration at half-maximal Ca^{2+} transient amplitude (FDHM, $F(2, 222) = 8.77, p < 0.001$, Fig. 6D) in the hiPSC-CM. We further confirmed that both 3D reshaping and BIN1 overexpression also significantly accelerated spontaneous Ca^{2+} transients at the level of Ca^{2+} release and reuptake in the second hiPSC-CM line (Figure SIV). Interestingly, cuboid BIN1-overexpressing hiPSC-CM revealed the best synchronised Ca^{2+} transients compared to any other conditions whereas remarkable delays of Ca^{2+} release were observed in the corresponding control groups (Fig. 6A, please refer to the zoomed images). In line with these observations, time-to-peak (TTP, Fig. 6E), decay (Fig. 6F) and FDHM (Fig. 6G) of stimulated Ca^{2+} transients were also significantly shortened in 3D-reshaped BIN1-overexpressing hiPSC-CM, particularly in the cuboid cell group.

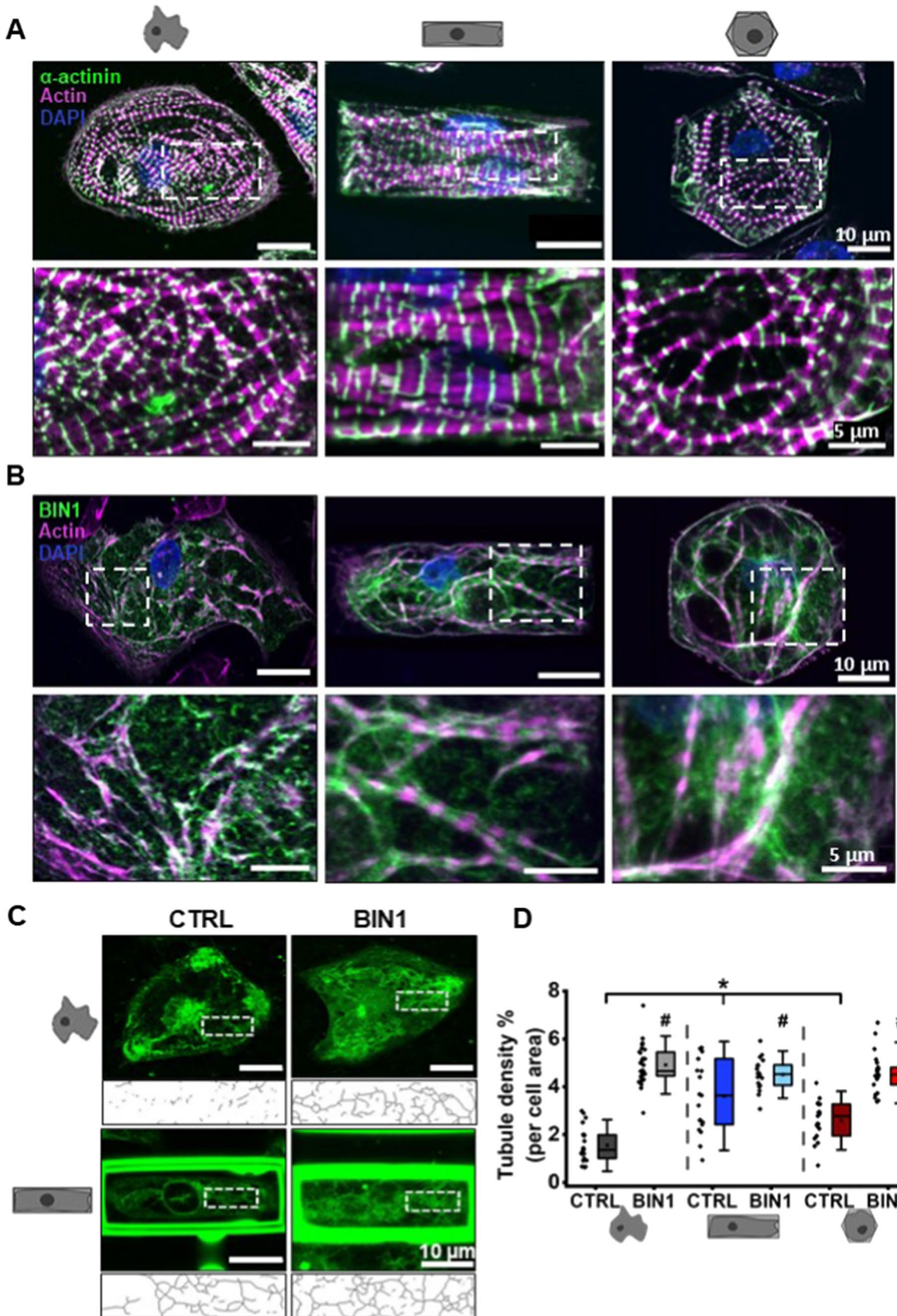


Fig. 2 3D reshaping and BIN1 overexpression lead to adaptations in cell morphology and microarchitecture. Immunolabeling of α -actinin (green, **A**), BIN1 (green, **B**), actin (magenta) and DNA (DAPI, blue) in BIN1-expressing, non-patterned, cuboid and hexagonally shaped hiPSC-CM. White boxes indicate the area of magnification. **C** Representative images of confocal live cell imaging of the sarcolemma using di-8-ANEPPS to visualise the tubular membrane network. White boxes illustrate the skeletonized images of the analysed regions of interest (ROI). **D** Statistical analysis of the tubule density within the ROIs in different experimental groups of hiPSC-CM. Two-way ANOVA was conducted to examine the synergistic effect of BIN1 expression and 3D reshaping on tubule density. $N=4$, $n=19-31$ cells; *indicates comparison between different shapes, and # between BIN1-overexpressing and shape control cells; $p < 0.05$. Data are presented as a box plot and whiskers show SD

Excitation–contraction coupling gain is improved in cuboid BIN1-overexpressing hiPSC-CM

Since we observed that Ca^{2+} transients were faster in 3D-reshaped BIN1-overexpressing hiPSC-CM, we also investigated EC coupling in more detail by analysing the EC coupling gain. This gain provides an assessment of the coupling fidelity between LTCCs and RyR2s. L-type Ca^{2+} currents (I_{CaL}) and current-stimulated Ca^{2+} transients were measured simultaneously with the following stimulation protocol: I_{Na} was inactivated by a voltage ramp from the holding potential (V_{H}) of -80 mV to -40 mV, then Ca^{2+} release was triggered by two separate voltage steps to -25 mV and $+10$ mV (please see voltage protocol in Fig. 7A). The EC coupling gain was calculated from the ratio of the peak Ca^{2+} transient amplitude and the corresponding peak I_{CaL} at -25 mV. The maximal I_{CaL} and Ca^{2+} release amplitude at $+10$ mV were measured for internal control. Interestingly, in 3D-reshaped and BIN1-overexpressing hiPSC-CM, distribution of the EC coupling gain moved towards higher values as compared to control non-patterned hiPSC-CM indicating improvement of coupling and, therefore, more efficient CICR (Fig. 7B). Moreover, two-way ANOVA revealed that both 3D reshaping and BIN1 overexpression have statistically significant effects on the EC coupling gain; however, no significant synergistic effects were observed ($F(2, 135) = 2.435$, $p = 0.091$, Fig. 7B). In conclusion, these experiments demonstrate that BIN1-overexpressing hiPSC-CM grown in cuboid micro-scaffolds develop better EC coupling leading to a significantly larger amplification of the I_{CaL} -triggered Ca^{2+} release compared to non-patterned control cells (Fig. 7B).

Finally, dependence of Ca^{2+} release from the filling state of the SR was determined in 1 Hz-stimulated patch-clamped cells. Fractional release of Ca^{2+} from the SR was determined by normalising the peak amplitude of paced Ca^{2+} transients to the peak amplitude of caffeine-induced Ca^{2+} transients (Fig. 7C, D). At constant triggering, on average 50% of the SR Ca^{2+} content was released per twitch, indicating similar

Ca^{2+} release properties and SR Ca^{2+} loads in the different groups of hiPSC-CM (Fig. 7D, E). This was further reflected by similar activities of the NCX in these cells assessed as integrated inward membrane currents ($\int I_{\text{NCX}}$) during prolonged caffeine application (Fig. 7F). The expression pattern of the two major proteins for Ca^{2+} removal, i.e. the reuptake of Ca^{2+} into the SR via SERCA2 and the extrusion of Ca^{2+} through the NCX, in non-patterned and 3D-reshaped BIN1-overexpressing hiPSC-CM is shown in Figure SV. SERCA2 expression revealed a dense network lining sarcolemmal BIN1 expression (Figure SVA) indicative of a tight association of the SR and the sarcolemma in BIN1-overexpressing hiPSC-CM. In contrast, NCX staining revealed a dotted distribution pattern over the entire sarcolemma (Figure SVB).

A comparison of the presented data is summarised in Table 1 to illustrate the specific effects of shape (cuboid/hexagon), BIN1 expression and combined effects on hiPSC-CM structure and function compared to non-patterned control cells.

Discussion

Despite their cardiogenic properties, hiPSC-CM differ from adult cardiomyocytes in many structural and functional details, which are generally summarised and connoted as immature features. The term immaturity in this context is inspired and derived from the immature characteristics of prenatal or neonatal cardiomyocytes. Despite distinct differences in the development, differentiation and properties of native or stem cell-derived cardiomyocytes, the concept of immaturity conveys the idea that, in compliance with native cells, further maturation of hiPSC-CM towards an adult phenotype is possible. Since the immaturity of hiPSC-CM limits their application for clinical purposes, current research focuses intensively on the development of efficient maturation strategies to achieve an adult-like phenotype that may be best suited for a cell-based therapy of the failing heart.

In this study, we focussed on the combined effects of changes in cell shape towards more adult morphology and molecular enhancement of BIN1 expression on Ca^{2+} handling and, thus, the basis of EC coupling in hiPSC-CM. In line with previous work [46], our data demonstrate that 3D reshaping induces structural reorganisation at the level of the subcellular microarchitecture in hiPSC-CM. Cuboid cells display parallel-aligned myofibrils, well-organised sarcomeres and a tubular membrane network reminiscent of early t-tubules. Since hiPSC-CM not only attach to the bottom surface but also to the walls of the scaffolds, resulting in an increase in cell height [46], the additional mechanical strain on the cell membrane may play a role in t-tubule biogenesis in 3D-reshaped hiPSC-CM, for which the triggering mechanism remains to be elucidated. In this context, passive

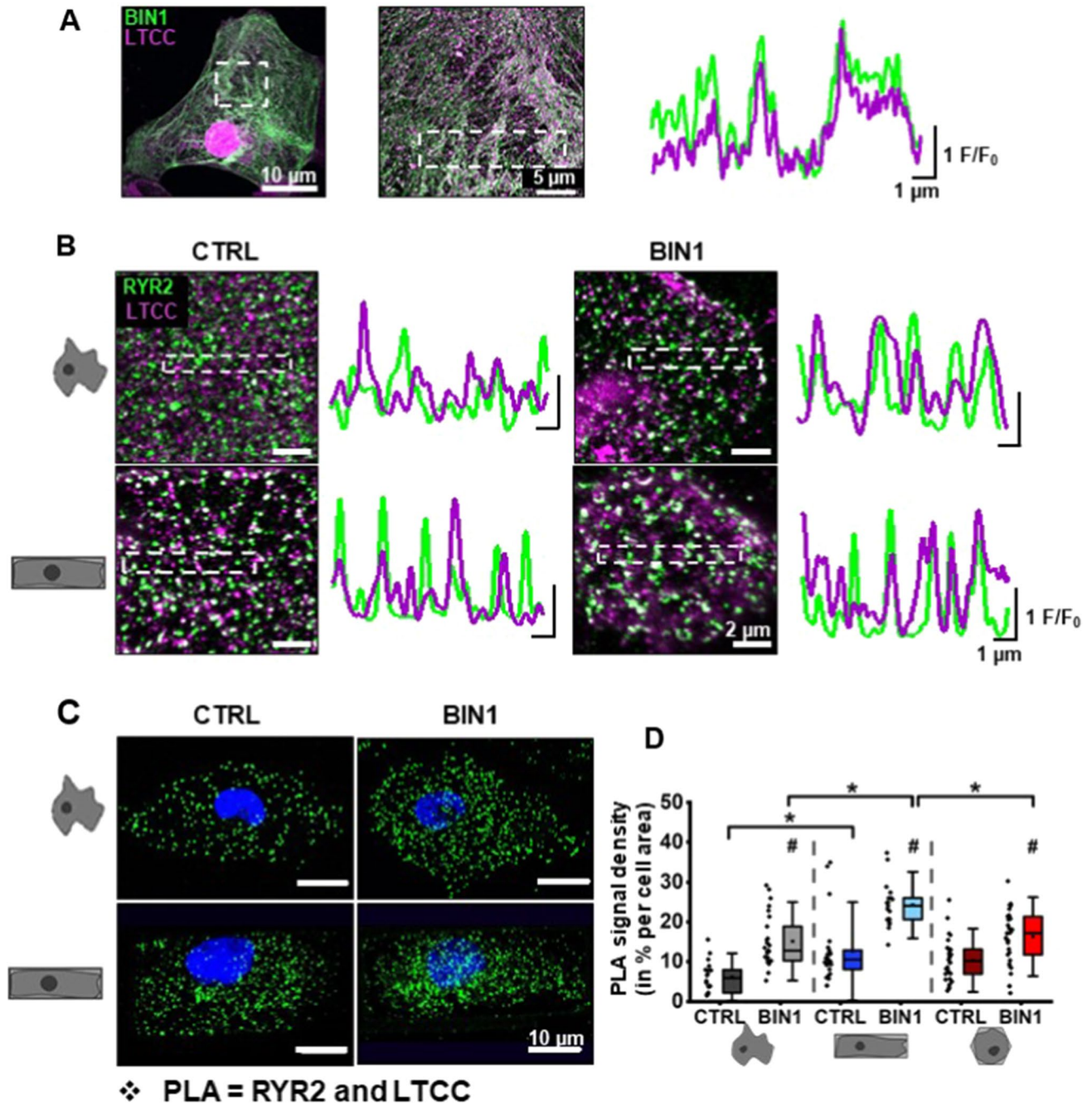


Fig. 3 Membrane remodelling reorganises the expression pattern of Ca^{2+} handling proteins and induces dyad formation. **A** Representative confocal images of the expression pattern of BIN1 (green) and LTCC (magenta) in a BIN1-expressing hiPSC-CM (left image; centre image: magnification of the ROI) and intensity profiles of the depicted ROI in the centre image. **B** Immunolabelling of RYR2 (green) and LTCC (magenta) and intensity profiles from the ROIs (white boxes) demonstrating spatial alignment of both ion channels relative to each other. **C** Representative confocal images from PLA:

green signals indicate the sites of interaction between RYR2 and LTCC in dyad microdomains (<40 nm). Nuclei are stained in blue. **D** PLA signal density was measured as a fraction of cell area occupied by fluorescence signals. Two-way ANOVA was conducted to examine the effect of BIN1 expression and 3D reshaping on LTCC-RyR2 cluster density. $N=4$, $n=18-30$ cells; * indicates comparison between different shapes; # indicates comparison between BIN1-overexpressing and shape control cells; $p < 0.05$. Data are presented as a box plot and whiskers show SD

resistance of the walls of the scaffold in conjunction with membrane stretch during contraction may activate mechanosensitive pathways such as, e.g. ion channels of the TRP

family [56], piezo channels [21, 60] and/or integrin-dependent outside-in signalling pathways [20] to elicit specific gene programmes for membrane remodelling.

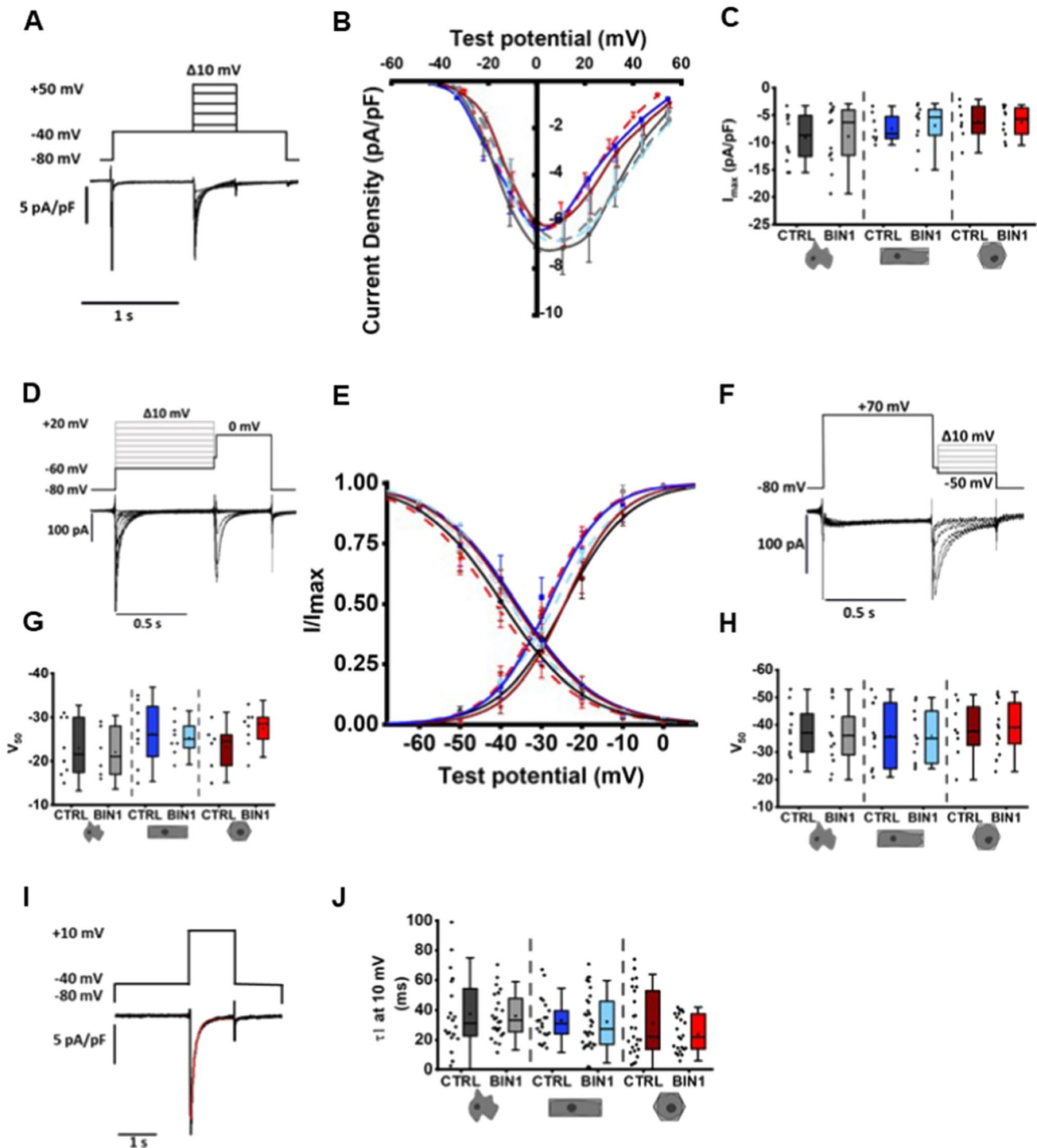
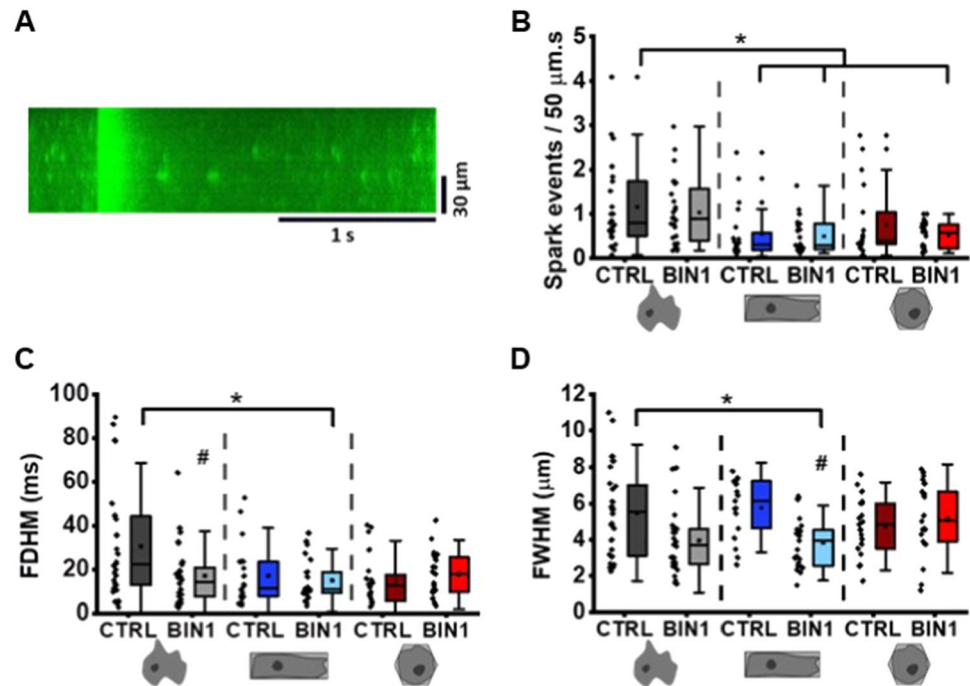


Fig. 4 Characterisation of I_{CaL} properties in reshaped BIN1-overexpressing hiPSC-CM. **A, D, F, I** Representative I_{CaL} traces triggered by the indicated voltage protocols. **B** Current–voltage relationship. **E** voltage-dependent steady-state activation and inactivation curves. **C** Analysis of peak current (I_{max} , $N=4-5$, $n=7-13$ cells). **G, H** Analysis of half-maximal voltage-dependent ($V_{1/2}$) activation and inactivation

($N=4-5$, $n=6-13$ cells). **I, J** Analysis of the time constant of the Ca^{2+} -dependent inactivation of I_{CaL} (τ_1) at +10 mV fitted with a bi-exponential function ($N=7-8$, $n=20-32$ cells). Two-way ANOVA revealed no significant differences between the experimental groups. Data are presented as a box plot and whiskers show SD

Fig. 5 Characterisation of Ca^{2+} spark events in reshaped BIN1-overexpressing hiPSC-CM. **A** Representative line-scan image of a spontaneous Ca^{2+} transient and Ca^{2+} sparks. Analysis of the number of spark events per 50 μm and second (**B**), full duration at half maximum (FDHM, **C**) and full width at half maximum (FWHM, **D**) in the different groups of hiPSC-CM. Statistical differences were tested by two-way ANOVA ($N=3$, $n=17-30$ cells); *indicates comparison between different shapes; # indicates comparison between BIN1-overexpressing and shape control cells; $p < 0.05$. Data are presented as a box plot and whiskers show SD



In addition to morphological remodelling by changing cell shape, we also overexpressed BIN1 in the hiPSC-CM. Previous reports suggested a possible role of BIN1 in the generation of membrane tubular networks [14, 41, 52]. Our findings demonstrate a strong induction of membrane invaginations by BIN1 overexpression already in non-patterned control hiPSC-CM, and even more so in cuboid cells, revealing a new level of membrane remodelling. Since BIN1 serves as a local anchor for LTCCs to stabilise their expression and localization at t-tubules [17], t-tubule formation is strictly followed by a subcellular rearrangement of LTCCs and RyR2s. The localization of LTCCs and RyR2s switched from a diffuse distribution to a more organised and striated expression pattern in cuboid BIN1-overexpressing hiPSC-CM leading to increased formation of dyads, i.e. subcellular microdomains where LTCCs and RyR2s are enriched for better functional interaction [11, 3]. In contrast to non-patterned control cells, the distance between both types of Ca^{2+} channel was reduced to less than 40 nm in 3D-reshaped BIN1-overexpressing hiPSC-CM, reminiscent of the narrow dyadic cleft of adult, i.e. mature cardiomyocytes. This close vicinity is indispensable for the functional interaction of LTCCs and RyR2s via rapid diffusion of Ca^{2+} ions, thus ensuring efficient EC coupling.

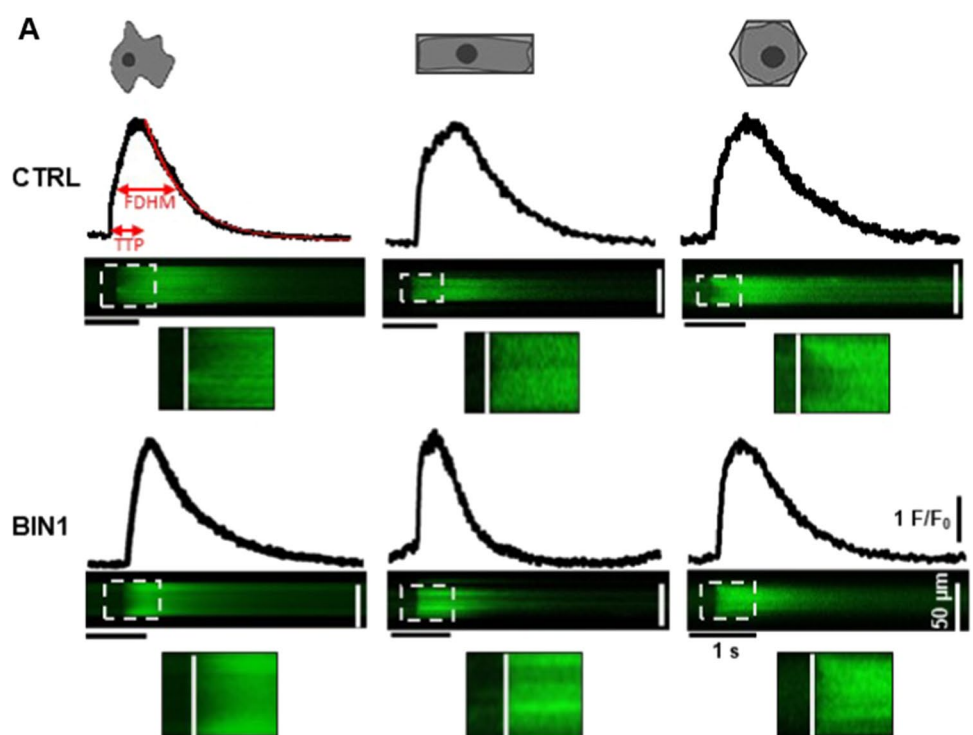
One hallmark of immaturity in hiPSC-CM is their spontaneous contractile activity. Although varied mechanisms underlying this autorhythmicity have been hotly debated, the interplay between so-called membrane and Ca^{2+} clock mechanisms may provide a plausible explanation. At the molecular level, hiPSC-CM expressed all essential Ca^{2+}

handling proteins contributing to Ca^{2+} influx, release and removal [61] to a similar degree in non-patterned and 3D-reshaped cells. While the membrane clock depends on the alternating activity of sarcolemmal ion channels, spontaneous Ca^{2+} release from the SR is key to the Ca^{2+} clock mechanism [26, 34, 53]. Therefore, we measured Ca^{2+} currents and both spontaneous and Ca^{2+} -triggered Ca^{2+} release events, respectively.

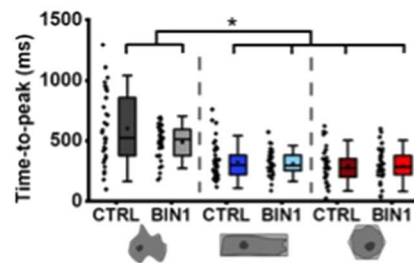
Electrophysiological evaluation of the LTCC-dependent Ca^{2+} current revealed no difference in its properties and kinetics among the different groups of hiPSC-CM, but Ca^{2+} signalling differed at the functional level pointing to a more mature Ca^{2+} handling in the cytoplasm. One indicator for this in structurally remodelled hiPSC-CM was the reduction in spontaneous Ca^{2+} release events from the SR. While non-patterned control cells revealed a high frequency of Ca^{2+} sparks and related spontaneous Ca^{2+} release events at resting conditions, their occurrence was significantly decreased in cuboid BIN1-overexpressing hiPSC-CM. Detailed analysis of spontaneous and Ca^{2+} -triggered Ca^{2+} release events revealed faster Ca^{2+} transients in 3D-remodelled cells irrespective of the shape both at the level of Ca^{2+} release from the SR (TTP) and Ca^{2+} reuptake via SERCA2 and extrusion via the NCX (FDHM), which is in accordance with previous studies of either BIN1-overexpressing or 3D-reshaped iPSC-CM [16, 46].

In dysfunctional cardiomyocytes originating from different models of cardiac disease [1, 13], loss of the t-tubular network and significantly decreased dyadic units are highly prevalent. Moreover, cardiomyocytes in which t-tubule

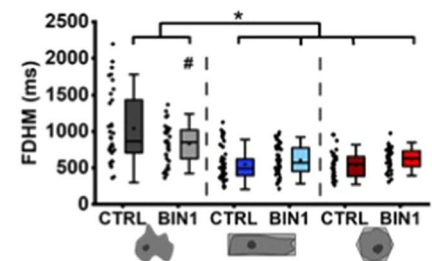
Fig. 6 Maturation of Ca²⁺ transient dynamics in 3D-reshaped BIN1-overexpressing hiPSC-CM. **A** Representative line-scan images and plot profiles of spontaneous Ca²⁺ transients in the different experimental groups. Analysis of time-to-peak (TTP; **B, D**), and full duration half maximum (FDHM; **C, E**) of spontaneous and stimulated Ca²⁺ transients, respectively. Statistical differences were tested by two-way ANOVA (*N* = 3–4, *n* = 33–49 cells); *indicates comparison between different shapes; #indicates comparison between BIN1-overexpressing and shape control cells; *p* < 0.05. Data are presented as a box plot and whiskers show SD



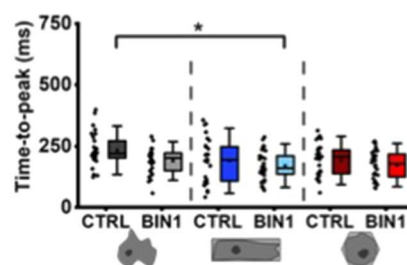
B Spontaneous Ca²⁺ transients



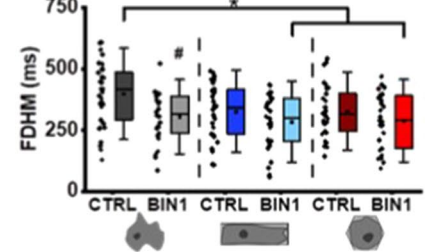
C



D Stimulated Ca²⁺ transients



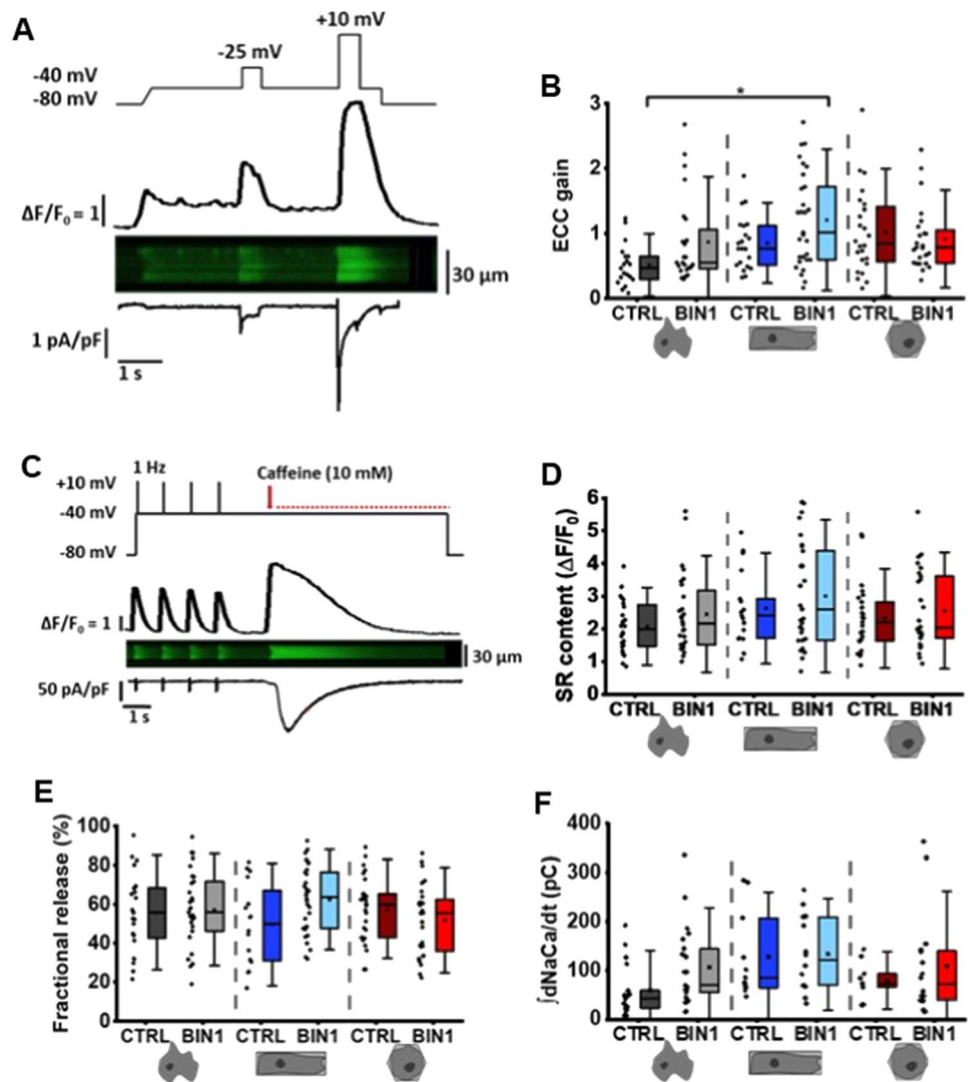
E



abundance is reduced reveal a decline in both BIN1 and LTCC abundance and significantly delayed Ca²⁺ transients [16]. Furthermore, the maladaptive remodelling processes leading to heart failure generally result in abnormal Ca²⁺ handling and specifically in desynchronised Ca²⁺ transients in the affected cardiomyocytes with the consequence of severely reduced EC coupling and decreased contractile

force [32, 57]. Hence, a well-structured tubular membrane network and the formation of functional dyads are essential for robust Ca²⁺ handling in cardiomyocytes, and therefore represent a major goal to achieve in hiPSC-CM [29]. In non-patterned control hiPSC-CM, the early phase of the Ca²⁺ transient, which is initiated by diffusion-dependent activation of the RyR2s via Ca²⁺ influx through the LTCCs,

Fig. 7 EC coupling gain is improved in cuboid BIN1-expressing hiPSC-CM. **A** Voltage protocol for I_{CaL} stimulation (upper panel), Ca^{2+} transients with corresponding line-scan image and line profile (middle panel) and recordings of I_{CaL} (lower traces). **B** EC coupling gain measured at -25 mV ($N=7-8$, $n=19-29$ cells) and distribution curves. **C** Stimulation protocol (upper panel), Ca^{2+} transient (line-scan and line profile, middle panel), and current traces (lower traces) to measure fractional release. Evaluation of **(D)** SR content, **E** fractional release and **F** NCX activity in hiPSC-CM ($N=5-6$, $n=9-24$ cells). Statistical comparison by two-way ANOVA. Data are presented as a box plot and whiskers show SD. *indicates comparison between different shapes; # indicates comparison between BIN1-overexpressing and control cells; $p < 0.05$



revealed strong desynchronization at the onset of the Ca^{2+} transients. This pattern can be best explained by inward propagation of the released Ca^{2+} from the peripheral cell membrane towards the cell centre rather than true CICR. In contrast, cuboid BIN1-expressing hiPSC-CM demonstrated a spatio-temporally synchronised onset of Ca^{2+} release from the SR. Due to the many BIN1-induced membrane invaginations, LTCCs are also located deep in the centre of the cell. As a consequence, sarcolemmal depolarization and LTCC-mediated Ca^{2+} influx lead to local CICR via junctional RyR2s simultaneously throughout the entire cell and not only at the cell periphery.

This improvement of junctional CICR was further corroborated by the significantly enhanced EC coupling gain in cuboid 3D-reshaped, BIN1-overexpressing hiPSC-CM. Our data demonstrate that despite similar levels of expression of the Ca^{2+} channels and Ca^{2+} influx currents, Ca^{2+} release from the SR was significantly greater in these cardiomyocytes due to shorter Ca^{2+} diffusion distances and, thus, a

more effective EC coupling gain. This increased gain is an important measure for amplification of the cytosolic Ca^{2+} signal through RyR2-mediated Ca^{2+} release from the SR and, therefore, confirms that structural remodelling leads to robust EC coupling, indicative of important functional maturation processes at the level of single hiPSC-CM.

In summary, our data provide strong evidence that structural remodelling of hiPSC-CM at the level of cell morphology and membrane organisation leads to optimization of the functional interaction between LTCCs and RyR2s, the key players of EC coupling in cardiomyocytes. In this context, the role of BIN1 as important steering wheel for membrane invaginations and consequently for the development of a more mature Ca^{2+} handling machinery suggests that BIN1 may also represent a promising target for the treatment of heart failure. Potential therapeutic effects of BIN1 gene therapy have already been tested at the preclinical level with preliminary success [31, 63]. With regard to hiPSC-CM-based cell replacement therapy, the combination of

Table 1 Summary of data comparison and illustration of additive effects: red boxes represent no significant differences compared to non-patterned control, light green boxes indicate statistically significant effects, dark green boxes indicate additive effects of particular shape and BIN1 expression

	Cuboid	Hexagon	BIN1	Cuboid+BIN1	Hexagon+BIN1
Myofibril alignment	✓	–	–	✓	–
t-tubule density	✓	✓	✓	✓	✓
PLA LTCC-RyR2	✓	–	✓	✓	✓
LTCC properties	–	–	–	–	–
Ca ²⁺ sparks freq	✓	–	–	✓	✓
Ca ²⁺ sparks FDHM	–	–	✓	✓	–
Ca ²⁺ sparks FWHM	–	–	–	✓	–
Spontaneous Ca ²⁺ transients: TTP	✓	✓	–	✓	✓
Spontaneous Ca ²⁺ transients: FDHM	✓	✓	✓	✓	✓
Triggered Ca ²⁺ transients: TTP	–	–	–	✓	–
Triggered Ca ²⁺ transients: FDHM	–	–	✓	✓	✓
EC coupling gain	–	–	–	✓	–
SR Ca ²⁺ content	–	–	–	–	–
Fractional release	–	–	–	–	–
NCX current	–	–	–	–	–

cardiomyocyte-specific BIN1 induction and tissue engineering approaches employing micropatterning of cells [6] may advance the development of cardiac patches and enhance the therapeutic potential of these cells for the treatment of patients with heart failure.

Limitations of the study

Here, we have addressed different levels of structural remodelling in the context of the rather complex development of fully mature cardiomyocytes from pluripotent stem cells that is in addition accompanied by some technical limitations. Our experimental model served primarily the identification of specific cues and features that are needed to drive hiPSC-CM to further maturation. In this context, one significant success in the maturation process is already a reduction in variability, as seen from our data. In the presented form, the tool of reshaping is not suited for in vivo experimentation, but importantly, the gain of knowledge that both longitudinal shape and t-tubule formation are required for functional maturation, will have to be considered for the preparation of cell grafts. In future studies, the impact of the composition of the extracellular matrix (ECM) and its stiffness [25] as well as the effect of outside-in signalling [23] on the functional maturation of these cells must be taken into account. More physiological growth surfaces may also permit prolonged culture times and, thus, a more effective functional maturation [48] of these cells. Therefore, it will be interesting to expand our

newly developed experimental model for reshaping single cells and analysing EC coupling properties to multicellular hiPSC-CM preparations with similar cellular characteristics. Instead of BIN1 overexpression by molecular engineering, it will be interesting to find more natural mechanisms to enhance endogenous BIN1 levels not only for the functional maturation of hiPSC-CM but also for the treatment of diseased adult cardiomyocytes with reduced t-tubules and EC coupling.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00395-023-00984-5>.

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Author contributions NDU conceived the study. NDU and FK designed the experiments, interpreted the data, generated the figures and wrote the manuscript. FK and KP performed the experiments and analysed the data. MM contributed to data analysis, edited the manuscript and generated the graphical abstract. EDL and MB produced the scaffolds. KR and DG designed and produced the AAV6. NDU, ML and MH critically reviewed and edited the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest None to declare.

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