

# Hyperfused stress-resistant mitochondria in E161K LMNA mutation associated with dilated cardiomyopathy

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## Research Article

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# Abstract

Mutations in lamin A/C (*LMNA*) are responsible for a broad spectrum of diseases, often affecting cardiac tissue. Lamin A/C is a crucial nuclear envelope protein involved in gene expression regulation, mechanosensing, and nuclear transport. *LMNA* mutations can cause severe cardiac dysfunctions like dilated cardiomyopathy, atrial or ventricular fibrillation, or cardiac failure. Since effective therapeutic options remain lacking, understanding the pathophysiological mechanisms and identifying protective interventions are essential.

In our study, we found that cardiomyocytes derived from induced pluripotent stem cells carrying the missense *LMNA* (p.Glu161Lys, E161K) mutation exhibit mitochondrial hyperfusion, as evidenced by an increased number of mitochondrial junctions and mitochondria per se, as well as mitochondrial elongation. Moreover, the hyperfused mitochondrial network was associated with basal hyperpolarisation and was more resistant to locally induced oxidative stress. The hyperfused network could be an adaptation to a stress situation due to an *LMNA* mutation, associated with increased ER stress, and may simultaneously provide protection against acute oxidative stress. Our results add to the previously described effects of *LMNA* mutations at the mitochondrial level, which underscore the important mitochondrial contribution in *LMNA*-deficient dilated cardiomyopathy.

## Introduction

Mutations in the gene encoding lamin A/C (*LMNA*) cause a variety of phenotypically heterogeneous diseases with the most prevalent cardiac involvement, including Emery-Dreifuss muscular dystrophy, limb-girdle muscular dystrophy 1B and extremely rare Hutchinson-Gilford Progeria Syndrome [1]. The most frequent *LMNA* missense and truncating mutations cause 5–8% of genetic dilated cardiomyopathy linked predominantly to sinus node and atrioventricular node dysfunction, atrial and/or ventricular fibrillation or heart failure [2]. Understanding the pathological mechanisms and identifying potential compounds to protect against *LMNA*-deficient dilated cardiomyopathy (LMNA-DCM) are essential, given the current lack of effective treatments for the disease.

Lamin A/C is an intermediate filament protein forming the nuclear lamina, a fibrous network underlining the inner nuclear membrane, which is important for processes such as gene expression regulation, mechanosignaling, DNA replication, or nuclear transport [3]. The expression of lamin A/C is higher in differentiated cells and further increased in tissues with higher stiffness and mechanical load [4]. The prevailing hypothesis is that *LMNA* mutations destabilise the nuclear lamina, leading to increased susceptibility to mechanical stress and overall cellular damage [5], although the cellular phenotype could be diverse. Nuclear blebbing or destabilisation is often observed [6–8], but in some cases, nuclear structure remains unaltered [9] when the *LMNA* mutation is present. Electrophysiological disturbances in patients with *LMNA* mutations are often associated with abnormal calcium handling [10–12].

Growing evidence indicates that mitochondrial dysfunction contributes significantly to heart failure and dilated cardiomyopathy [13]. The *LMNA* mutations were associated with defective mitochondrial cristae

[9], impaired mitochondrial bioenergetics [12, 14], elevated reactive oxygen species (ROS) and oxidative stress [12] in the heart. Elevated ROS levels and intracellular calcium concentration can trigger mitochondrial oscillations or even depolarisation, creating a metabolic sink, thereby elevating the arrhythmogenic substrate via activation of ATP-sensitive potassium channels [15]. Additionally, *LMNA* mutations cause the accumulation of misfolded lamin A/C in the endoplasmic reticulum (ER) or in the nucleus, leading to ER stress and activation of the unfolded protein response (UPR), which can activate C/EBP homologous protein (CHOP) cascade, eventually promoting the upregulation of proapoptotic genes [16–18]. All these cellular dysfunctions create a vicious circle leading to cardiac dysfunction and failure.

In our study, we found that *LMNA*-deficient (c.481G > A, p.Glu161Lys, E161K) ventricular cardiomyocytes derived from induced pluripotent stem cells (iPSC-CMs) [19] have increased mitochondrial hyperfusion associated with basal mitochondrial hyperpolarisation and better mitochondrial survival upon acute oxidative stress. This may result from adaptation to ongoing ER stress.

## Materials and methods

### iPSC culture and *in vitro* cardiac differentiation

Human induced pluripotent stem cells (iPSCs) generated from a patient with a heterozygous *LMNA* variant [19] (line EURACi015-A, c.481G > A, p.Glu161Lys, E161K) and gender- and age-matched iPSCs commercially available (SCTI004\_A, StemCell Technologies) were used in this study. The experiments involving both iPSC lines were performed in accordance with the Declaration of Helsinki, and all cell culture procedures were in line with the national biosafety standards of Slovakia. The cell line EURACi015-A was generated in the lab of M. de Bortoli [19] where the donor's written consent was obtained. The EURACi015-A was kindly gifted by S. Zacchigna.

The patient carrying a heterozygous pathogenic missense variant of the *LMNA* gene was affected by a severe familial form of dilated cardiomyopathy, with a heart failure diagnosis and life-threatening ventricular arrhythmias [19]. The iPSC lines were cultured on Geltrex™ (Thermo Fisher Scientific)-coated plates in STEM-FLEX medium (Thermo Fisher Scientific) according to the manufacturer's protocol. When cells reached a confluence of 80–90%, they were detached using Accutase enzyme (Thermo Fisher Scientific), centrifuged at 200 g for 4 min, resuspended in culture medium supplemented with 10 µM Rock Inhibitor (Thiazovivin, Sigma-Aldrich) for 24h, and plated in a new Geltrex-coated plate. Cells were stored at 37°C in 5% CO<sub>2</sub> and routinely tested for mycoplasma and karyotype.

To produce iPSC-derived ventricular cardiomyocytes, we used the established protocol [20]. When iPSCs reached 70–80% confluency, the medium was replaced with RPMI/B27 without insulin (Thermo Fisher Scientific), supplemented with 6 µM GSK3-β inhibitor CHIR99021 (BLDpharm), and the cells were maintained for 48 h. Then, the medium was replaced by RPMI/B27 without insulin for 24 h. On day 3, RPMI/B27 without insulin, with 5 µM Wnt inhibitor IWR1 (MedChemExpress), was replaced and maintained for 48 h. The medium is changed back to RPMI/B27 without insulin medium for 48 h on day

5. Change to RPMI/B27 medium with insulin is on day 7 and maintained for 3 days when spontaneous beating should occur. The population of cardiomyocytes was purified via a metabolic switch by switching to low-glucose RPMI from day 10 for 3 days. Then, cells are returned to RPMI/B27 medium with insulin for 3 days. A second glucose starvation is initiated on day 14, with low-glucose medium added for 3 days. The remaining cells are highly purified cardiomyocytes, maintained in RPMI/B27 medium with insulin until days 21–25.

Mitochondrial morphology, basal mitochondrial membrane potential ( $\Delta\Psi_m$ ) & ROS level

$3 \times 10^5$  iPSC-derived CMs were seeded in RPMI/B27 with insulin medium in 35 mm Glass bottom MatTek Petri dishes. After 48 h, the cells were loaded for 30 min at 37°C in the dark with 100 nM Mitotracker Deep Red (Invitrogen) and 100 nM probe tetra-methyl rhodamine methyl ester (TMRM) in non-quenching mode (Invitrogen) in the same medium. The cells were then placed in prewarmed medium.

Fluorescent images were acquired using a Leica SP8 STED 3X confocal microscope with an oil-immersion 63x objective, zoom 3, pixel format 1048 x 1048 and a scanning speed of 700 Hz. The excitation/emission wavelengths were set to TMRM: 552 nm/557–642 nm; MitoTracker Red: 641 nm/646–784 nm. The mitochondrial network was analysed in Fiji[21] using the *Mitochondria Analyzer* plugin [22]. Specifically, we used the weighted mean method with a block size of 1.35  $\mu\text{m}$  and a C-value of 13 to determine the local threshold in images.

Based on thresholded mitochondrial images, we created masks containing regions of interest (ROIs) to evaluate TMRM fluorescence as the mean grey value (in AU) per mitochondrion under basal conditions and to estimate basal mitochondrial potential as the mean of these values. These masks were also used in subsequent experiments involving acute oxidative stress.

We analysed the transmission and corresponding fluorescence images of the cells (zoom 1.28, pixel format 2048 x 2048) to determine cell size and evaluate possible hypertrophy. We evaluated cell areas and major and minor axes after fitting ellipses to whole cell ROIs in calibrated images using Fiji software.

Acute oxidative stress induced by laser irradiation

To induce the acute photodamage (PDM) by laser irradiation, the small cell area (app. 10  $\mu\text{m}^2$ ) containing two to five mitochondria was exposed to 405 nm laser flash at 100% power for 66 s. PDM-induced oxidative stress causes depolarisation of target mitochondria and local ROS generation, allowing us to examine a propagating effect from the affected area to surrounding mitochondria, depending on their distance. We used the fluorescence recovery after photobleaching module in the LASX software. Time-scanning was performed in a sequence of 10 control frames, followed by 30 frames in photodamage mode, and then a series of frames after photodamage (at least 90 images) until at least half of the mitochondria were depolarised.

The series of TMRM signals after PDM were analysed to obtain information on the present oscillations, which depend on the distance from the damaged area. The zone widths were set to 15  $\mu\text{m}$ . Then, the centroids of all ROIs were determined, and the calculation of the Euclidean distance determined the dynamic group assignment for all mitochondria/ROI.

Time-dependent TMRM signal oscillations were analysed to find the number of oscillations. The time dependence of the intensity of TMRM fluorescent dye was analysed using a script in GNU Octave 9.4. The raw data were smoothed using the Savitzky-Golay algorithm (second-order polynomial, window of 7 values). The first derivative of the smoothed data was computed, as at the peaks (either negative or positive), the first derivative is zero. The number of times the derivative changed sign from positive to negative was then determined. This value gives us the number of intensity maxima after the laser flash.

For the detection of mitochondrial depolarisation, the average and standard deviation from the first 10 points of recording were calculated (stable conditions prior to the laser flash) from the raw data. The rolling mean and standard deviation were calculated from the subsequent 10 points, each time moving one point forward. The mitochondrion was considered depolarised when the mean was  $< 0.2$  mean(basal) and SD reached the basal SD value. The time point at which this was achieved was set as the time of depolarisation, with zero time starting after the end of the laser flash. We used survival analysis to determine whether mitochondrial survival (i.e., the time to final mitochondrial depolarisation) differs between groups.

### Immunofluorescent labelling

The differentiated iPSC-CM cells were seeded on coverslips at a density of  $1-3 \times 10^5/\text{cm}^2$ . After 48 hours, the cells were fixed with ice-cold methanol for 5 min, washed with phosphate-buffered saline (PBS) and non-specific interactions were blocked with PBS supplemented with 5% bovine serum albumin and 0.1% Tween 20 for 1 hour at room temperature (RT). Afterwards, the cells were incubated with primary antibodies conjugated to a fluorophore for 1 hour at RT, followed by a PBS wash supplemented with 0.1% Tween 20. Finally, Fluoroshield with DAPI (Sigma-Aldrich) was used as a mounting medium. We used CoraLite®594-conjugated GRP78/BIP polyclonal antibody (CL594-11587, Proteintech) and CoraLite® Plus 488-conjugated CHOP/GADD153 polyclonal antibody (CL488-15204, Proteintech) in a dilution of 1:200 to see markers of ER stress in iPSC-derived cardiomyocytes.

### Reverse Transcription PCR

## Total RNA isolation

Mature iPSC-derived CMs ( $1-5 \times 10^6$  cells) were harvested as previously described. The pellets were rinsed twice in PBS (Biosera) and stored at  $-20^\circ\text{C}$  until the following day. Cell pellets were maintained on ice during the initial preparation. For total RNA extraction, TRIzol reagent (TRI Reagent, Sigma-Aldrich) was added to each cell pellet, followed by thorough homogenization by vortexing. We used chloroform (Slavus) for phase separation; the samples were then vortexed vigorously and incubated at room

temperature. By subsequent centrifugation ( $11,000 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ ), we separated the aqueous phase containing RNA from the total volume. RNA was precipitated by adding isopropanol (Centralchem) to the collected aqueous phase. Samples were gently mixed and incubated for 10 min at room temperature. RNA precipitation was performed by adding isopropanol (Centralchem), followed by incubation at room temperature. The precipitated RNA was centrifuged ( $11,000 \times g$  for 15 minutes at  $4^{\circ}\text{C}$ ), and after removing the supernatant, the pellets were washed with 75% ethanol. After removing the ethanol, the samples were allowed to dry at room temperature, then dissolved in 20  $\mu\text{L}$  of sterile, Ambion™ Nuclease-Free Water (Invitrogen) and stored at  $-80^{\circ}\text{C}$ .

RNA concentration and purity were determined spectrophotometrically (Nanophotometer, IMPLEN) by measuring absorbance at 260 and 280 nm. RNA purity was assessed based on the A260/A280 absorbance ratio.

All procedures were performed using RNase-free consumables and solutions, and laboratory surfaces were disinfected with alcohol prior to sample processing.

## Reverse transcription and cDNA synthesis

Complementary DNA (cDNA) was synthesised from the isolated total RNA (using 1  $\mu\text{g}$  of total RNA from each dilution) by reverse transcription using random hexamer primers and reverse transcriptase. For the initial denaturation step, random hexamer primer (ProtoScript® II First Strand cDNA Synthesis Kit, New England Biolabs), the appropriate volume of Ambion™ Nuclease-Free Water (Invitrogen), and the RNA sample were combined to a final volume of 12  $\mu\text{L}$ . The mixture was incubated at  $65^{\circ}\text{C}$  for 5 min in a thermocycler.

A reverse-transcription master mix was prepared containing 5 $\times$  Reaction Buffer, RiboLock RNase inhibitor, 10 mM dNTP mix, and reverse transcriptase per reaction (ProtoScript® II First Strand cDNA Synthesis Kit, E6560S). Following the initial denaturation, the RNA/primer mixtures were immediately placed on ice, and the reverse-transcription master mix was added to each sample, resulting in a final reaction volume of 20  $\mu\text{L}$ . Following completion of the reaction, the resulting cDNA was stored at  $-20^{\circ}\text{C}$  until further analysis or used immediately as a template for PCR amplification.

## Reverse transcription PCR (RT-PCR) and electrophoresis

Reverse transcription PCR (RT-PCR) and electrophoresis

The synthesised cDNA was used as a template for conventional PCR amplification of the genes of interest. PCR amplification was performed in a thermocycler according to the following cycling conditions: (1)  $95^{\circ}\text{C}$  polymerase activation for 5 min, followed by (2) 30 cycles consisting of (a) denaturation for 60 s at  $95^{\circ}\text{C}$  (b) annealing for 40 s (temperatures in Table 1) (c) elongation for 60 s at  $72^{\circ}\text{C}$ , and (3) final elongation 5 min at  $72^{\circ}\text{C}$ . PCR reactions were prepared using the appropriate PCR reaction mixture and gene-specific forward and reverse primers (Table 1).

The PCR products were separated by electrophoresis on a 1.5% agarose gel (Lonza) and visualised using GelRed™ nucleic acid gel stain (Biotium). Following electrophoresis, the separated PCR products were visualised using an Amersham Imager 600 imaging system (GE Healthcare Europe GmbH). Images were acquired using the instrument's appropriate nucleic acid detection settings. The resulting images were used for documentation and, where applicable, densitometric analysis of the amplified PCR products.

We monitored the stability of expression of several housekeeping genes (*GAPDH*, *RPL13A*, *B2M*, and *HPRT1*) due to reported changes in their expression during iPSC differentiation [23]. We observed stable expression only for the *B2M* gene, which later served as the reference gene for densitometric analysis of the genes under study.

Table 1  
**Primers, PCR annealing temperature, and product size.** Primer pairs were pre-designed and ordered from Merck (Sigma–Aldrich) with desalting purification. All primer pairs were tested experimentally with a temperature gradient.

| Gene            | Primer Sequences                   | T <sub>A</sub> (°C) | PCR Product (bp) |
|-----------------|------------------------------------|---------------------|------------------|
| <i>ATF4</i>     | F: 5'-CCTAGGTCTCTTAGATGATTACC-3'   | 56                  | 199              |
|                 | R: 5'-CAAGTCGAACTCCTTCAAATC-3'     |                     |                  |
| <i>B2M</i>      | F: 5'-AAGGACTGGTCTTTCTATCTC-3'     | 57                  | 118              |
|                 | R: 5'-GATCCCACTTAACTATCTTGG-3'     |                     |                  |
| <i>DENR</i>     | F: 5'-AAATTCCCAGAGCAAAGAAG – 3'    | 57                  | 164              |
|                 | R: 5'-GTCATCTGTAAAATCTCCCTG – 3'   |                     |                  |
| <i>HSPA5</i>    | F: 5'- TCTATGAAGGTGAAAGACCC-3'     | 58                  | 114              |
|                 | R: 5'- TCTCAAAGGTGACTTCAATC – 3'   |                     |                  |
| <i>HYOU1</i>    | F: 5'- CCGGAAAGATATTAACACCAC – 3'  | 58                  | 106              |
|                 | R: 5'- TTCCTTAGTCTTCACCATCTG – 3'  |                     |                  |
| <i>MFN1</i>     | F: 5'- ATCTTTGAGGAGTGTATCTCG – 3'  | 56                  | 83               |
|                 | R: 5'- GTAGCTAGTATCTGTTTAGCTC – 3' |                     |                  |
| <i>MFN2</i>     | F: 5'- CTTCTTTGTGTCTGCTAAGG – 3'   | 56                  | 115              |
|                 | R: 5'- CTGAAACTCAAACATCCTCAC – 3'  |                     |                  |
| <i>OPA1</i>     | F: 5'- TTAAGGTGACTCTGAGTGAAG – 3'  | 57                  | 106              |
|                 | R: 5'- TCATGTCTTAATGCTGCAAG – 3'   |                     |                  |
| <i>PPP1R15A</i> | F: 5'- ATCTATGTACCTGGAGAGAAG – 3'  | 57                  | 138              |
|                 | R: 5'- CACCTTTCTGGCCTTTAG – 3'     |                     |                  |

# Statistical analysis

All data were tested for normality (d'Agostino & Pearson Normality test). Statistical significance at the level  $P < 0.05$  was determined using the t-test for normally distributed data or the Mann-Whitney test for non-normally distributed data. Normally distributed data are described by the mean and the standard error of the mean (SEM) out of  $n$  values. Non-Normally distributed data are described by the median and interquartile range (IQR) from  $n$  values. Statistical analysis and graph preparation were performed in GraphPad Prism 10.4 software (GraphPad Software, San Diego, CA).

## Results

### Mitochondrial hyperfusion in *LMNA*-deficient cardiomyocytes

iPSC-derived cardiomyocytes from control line SCT1004 (ctrl) and line EURACi015-A (*LMNA*) with missense *LMNA* mutation (c.481G > A, p.Glu161Lys) were labelled with Mitotracker Deep Red to visualise mitochondrial networks. We found that control cardiomyocytes have thin mitochondria with the expected elongated shape, mostly separated, with occasional junctions (Fig. 1A, **left**). The mitochondria in *LMNA*-deficient cardiomyocytes were significantly longer and created more pronounced networks with a high number of junctions (**Fig. A right**). When we evaluated mitochondrial parameters ( $n$  (ctrl) = 48,  $n$ (*LMNA*) = 34), we observed a significantly increased total mitochondrial area in *LMNA*-deficient cardiomyocytes compared to controls (Fig. 1B; Mann-Whitney test: median (ctrl) =  $44.18 \mu\text{m}^2$ , median (*LMNA*) =  $88.01 \mu\text{m}^2$ ,  $p < 0.0001$ ). Moreover, the mitochondria in *LMNA*-deficient cardiomyocytes had significantly increased perimeter (Fig. 1C; Mann-Whitney test: median (ctrl) =  $3.81 \mu\text{m}$ , median (*LMNA*) =  $4.73 \mu\text{m}$ ,  $p < 0.0001$ ), were significantly elongated (Fig. 1D; Mann-Whitney test: median (ctrl) =  $2.47 \mu\text{m}$ , median (*LMNA*) =  $2.84 \mu\text{m}$ ,  $p = 0.0038$ ) and had more complex shape (Fig. 1E; unpaired t-test: mean (ctrl) = 2.32, mean (*LMNA*) = 2.69,  $p = 0.0039$ ) compared to controls. As expected, the network parameters were also significantly pronounced in *LMNA*-deficient cardiomyocytes, such as the ratio of branches per mitochondria (Fig. 1F; Mann-Whitney test: median (ctrl) = 0.08, median (*LMNA*) = 0.23,  $p < 0.0001$ ) and mean branch length (Fig. 1G; unpaired t-test: mean (ctrl) = 0.99, mean (*LMNA*) = 1.33,  $p < 0.0001$ ), compared to controls.

Mitochondrial dynamics and network properties are altered when the fusion-to-fission balance changes. The proteins essential for outer mitochondrial membrane fusion are Mitofusin 1 & 2 (*MFN1* & 2); Optic Atrophy 1 protein (*OPA1*) regulates the fusion of inner membranes and cristae organisation, whereas Dynamin-related protein 1 (*DRP1*) is associated mostly with fission [24]. Next, we wanted to check mRNA levels of the main mitochondrial dynamics' genes (*MFN1*, *MFN2*, *DRP1*, and *OPA1*) using semi-quantitative RT-PCR (Fig. 1H). We used *B2M* ( $\beta$ -2-microglobulin) as housekeeper gene. Densitometric analysis of gene expressions showed no significant change in *MFN1* level (Fig. 1H & I; unpaired t-test: mean (ctrl) = 0.57, mean (*LMNA*) = 0.8,  $p = 0.46$ ), *MFN2* level (Fig. 1H & J; unpaired t-test: mean (ctrl) = 0.65, mean (*LMNA*) = 0.74,  $p = 0.74$ ), *OPA1* level (Fig. 1H & K; unpaired t-test: mean (ctrl) = 0.5, mean

(LMNA) = 0.67,  $p = 0.5$ ) or *DRP1* level (Fig. 1H & L; unpaired t-test: mean (ctrl) = 0.58, mean (LMNA) = 0.51,  $p = 0.72$ ).

Taken together, mitochondrial hyperfusion and the formation of extensive mitochondrial networks were observed in *LMNA*-deficient cardiomyocytes compared with controls, although mRNA levels of proteins that regulate mitochondrial dynamics were not significantly altered.

### Hypertrophy of *LMNA*-deficient cardiomyocytes

We have hypothesised that mitochondrial enlargement may be associated with cellular hypertrophy in *LMNA*-deficient cardiomyocytes, which is also in line with the eccentric hypertrophy of the left ventricle occurring during *LMNA*-deficient dilated cardiomyopathy [6]. We evaluated the cell area and the major and minor axes after fitting an ellipsoid (Fig. 2A). We observed a significantly increased area in *LMNA*-deficient cardiomyocytes compared to controls (Fig. 2B; Mann-Whitney test: median (ctrl) = 957.8  $\mu\text{m}^2$ , median (LMNA) = 1613.0  $\mu\text{m}^2$ ,  $p = 0.0024$ ). Cells were enlarged in both directions: significantly increased major axis of ellipsoid (Fig. 2C; Mann-Whitney test: median (ctrl) = 51.97  $\mu\text{m}$ , median (LMNA) = 62.53  $\mu\text{m}$ ,  $p = 0.036$ ) mirroring growth in longitudinal direction as well as enlarged minor axis of ellipsoid (Fig. 2D; Mann-Whitney test: median (ctrl) = 24.01  $\mu\text{m}$ , median (LMNA) = 34.48  $\mu\text{m}$ ,  $p = 0.0008$ ) mirroring growth in transversal axis. We observed significant enlargement of *LMNA*-deficient cardiomyocytes compared to controls, although we could not determine whether the hypertrophy was eccentric or concentric.

### Changed mitochondrial potential in *LMNA*-deficient cardiomyocytes

The mitochondrial potential ( $\Delta\Psi_m$ ) results from redox transformations linked to the Krebs cycle and serves as an intermediate form of energy storage used by ATP synthase to produce ATP [25]. To assess mitochondrial function in our cells, we have measured  $\Delta\Psi_m$  using the fluorescent dye TMRM. We found significantly increased TMRM fluorescence (Fig. 3A; unpaired t-test: mean (ctrl) = 70.26, mean (LMNA) = 85.36,  $p = 0.004$ ) reflecting increased  $\Delta\Psi_m$ , suggesting overall mitochondrial hyperpolarisation at the basal conditions in *LMNA*-deficient cardiomyocytes compared to controls.

To challenge mitochondrial metabolism, we induced acute oxidative stress using laser-induced photodamage and observed changes in TMRM fluorescence /  $\Delta\Psi_m$ : basal  $\Delta\Psi_m$  starts oscillating after acute PDM, resulting in irreversible mitochondrial depolarisation (Fig. 1D). We evaluated the proportion of time-dependent mitochondrial depolarisation using survival analysis to estimate the functional mitochondrial survival rate between groups. We found that mitochondrial survival was significantly better (Fig. 3B; Log-rank (Mantel-Cox) test, median survival (ctrl) = 190.2s, median survival (LMNA) = 269.5s,  $p = < 0.0001$ ) in *LMNA*-deficient cardiomyocytes compared to controls. Acute oxidative stress is driven by ROS accumulation, which also promotes mitochondrial oscillations [26]. We separated the mitochondria into groups (near/  $<15 \mu\text{m}$ , middle/  $15\text{--}30 \mu\text{m}$ , and far/  $>30 \mu\text{m}$ ) based on their distance from the damaged area (Fig. 3C), and we evaluated the number of oscillations across groups. We found that the number of oscillations was similar in damaged, near and middle cellular areas, with significantly

fewer oscillations in the far area in the control group (Fig. 3E; one-way ANOVA,  $p = 0.0014$ : mean (damage) = 8.2, mean ( $< 15 \mu\text{m}$ ) = 8.32, mean ( $15\text{--}30 \mu\text{m}$ ) = 9.03; mean ( $> 30 \mu\text{m}$ ) = 5.04) as well as in the LMNA group (Fig. 3F; one-way ANOVA,  $p < 0.0001$ : mean (damage) = 12.33, mean ( $< 15 \mu\text{m}$ ) = 12.23, mean ( $15\text{--}30 \mu\text{m}$ ) = 11.74; mean ( $> 30 \mu\text{m}$ ) = 7.93). When compared among distance groups (Fig. 3G) we observed significant differences in oscillation number between distance groups as well as control vs. LMNA groups (two-way ANOVA, distance:  $p < 0.0001$ , patient/ctrl:  $p < 0.0001$ ).

Mitochondrial potential is higher in hyperfused mitochondria, indicative of altered mitochondrial turnover. Interestingly, the mitochondrial survival after severe oxidative stress is better in LMNA-deficient cardiomyocytes. These changes in  $\Delta\psi_m$  suggest activation of the pro-survival program and adaptation to possible chronic stress in LMNA-deficient cardiomyocytes.

### Increased ER stress in LMNA-deficient cardiomyocytes

Alterations in mitochondrial morphology are associated with ER stress; initially, reversible mitochondrial fragmentation is accompanied by increased calcium levels, followed by stress-induced mitochondrial hyperfusion, during which mitochondria become more elongated to support efficient ATP production, and finally, irreversible mitochondrial fragmentation triggers apoptosis as the last step of prevalent ER stress [27]. Our hypothesis was that observed mitochondrial hyperfusion and increased mitochondrial metabolism could be an adaptive response to ongoing ER stress. We used immunofluorescence labelling for acute and chronic ER stress; glucose-regulated protein 78 (GP78), also known as binding immunoglobulin protein (BiP), is a molecular chaperone protein that dissociates from its binding sites when an increased UPR signal is present as an acute ER stress marker [28], whereas CHOP is a main agent of ER stress-induced apoptosis used as a marker of chronic ER stress [29]. We found out a significantly increased level of GP78/BiP in LMNA-deficient cardiomyocytes (Fig. 4A **red** & Fig. 4C; Mann-Whitney test: median (ctrl) = 22.15 AU, median (LMNA) = 28.40 AU,  $p = 0.003$ ) compared to control. The CHOP expression was not significantly increased in the LMNA group compared to the control, although the trend of increased expression is present close to a significant threshold (Fig. 4A **red** & Fig. 4B; Mann-Whitney test: median (ctrl) = 6.51 AU, median (LMNA) = 7.36 AU,  $p = 0.0532$ ).

Then we assessed mRNA levels of genes involved in ER stress signalling by RT-PCR. However, the mRNA level of the *HSPA5* gene coding GP78/BiP was unchanged between the LMNA and the control group (Fig. 4D & Fig. 4E; unpaired t-test: mean (ctrl) = 1.09, mean (LMNA) = 1.28,  $p = 0.63$ ). A similar level of mRNA (Fig. 4D & Fig. 4F; unpaired t-test: mean (ctrl) = 1.4, mean (LMNA) = 1.2,  $p = 0.65$ ) was observed in both groups with the *HYOU1* gene coding calnexin, an ER chaperone, whose expression increases with GP78/BiP [30]. We have also evaluated UPR activation via the PERK signalling pathway: the gene encoding activating transcription factor 4 (ATF4) [28], which plays a role in activating the CHOP protein, and the gene encoding protein phosphatase 1 regulatory subunit 15A (*PPP1R15A*), which serves as a sensor for ER stress stimuli [31]. However, we did not observe significant differences in mRNA levels of genes ATF4 (Fig. 4D & Fig. 4G; Man-Whitney test: median (ctrl) = 0.82, median (LMNA) = 0.92,  $p = 0.39$ ) or

*PPP1R15A* (Fig. 4D & Fig. 4F; unpaired t-test: mean (ctrl) = 0.56, mean (LMNA) = 0.63,  $p = 0.71$ ) between control and *LMNA*-deficient cardiomyocytes.

We observed ER stress, possibly associated with UPR, marked by a significant increase in GRP78/BiP protein level in *LMNA*-deficient cardiomyocytes compared to controls. However, the mRNA levels of observed proteins associated with PERK signalling pathways were not significantly changed.

## Discussion

Currently, the clinical management of the *LMNA*-DCM is based on genotype–phenotype knowledge: the only amelioration of the clinical consequences so far related to the *LMNA* mutation is lowering of the age threshold for cardiac defibrillator implantation in patients with a *LMNA* mutation, which is related to a clinically higher risk of rhythm disturbances and sudden cardiac death [32]. **Exploring cellular pathologies could yield new targeted therapies.** In one case study, experimental results showed that increased mTOR signalling could be blocked by rapamycin, thereby ameliorating arrhythmia in a patient with an *LMNA* mutation [33]. Blocking of the p38 pathway by ARRY-371797 was shown to prevent chamber dilatation [34, 35], and led to a phase 3 clinical trial (NCT03439514) specific for *LMNA*-DCM, which was later terminated due to non-significant differences between groups [36].

In our study, we explored the iPSC-derived cardiomyocytes derived from a patient carrying the *LMNA* mutation c.481G > A, p.Glu161Lys (**E161K**) variant. It is a severe missense heterozygous mutation which was associated with life-threatening ventricular arrhythmias and heart failure [19]. The *LMNA* (E161K) mutation is well-known and has been reported in several studies of patients presenting with atrial fibrillation, left bundle branch block, and non-sustained ventricular tachycardia [37, 38]. The alteration of gene expression and mislocalisation of gene clusters at chromosome 13 were found in *LMNA* E161K hearts [39]. At the cellular level, this mutation is associated with mild cellular blebbing and cytoskeletal disorganisation [33, 35], nuclear lamina aggregation [40] and less-organised microtubule networks [41].

In our study, we focused on mitochondrial (dys)function in *LMNA*-DCM, given their essential role in cardiac function; a healthy mitochondrial population is crucial for cardiac homeostasis. One characteristic feature of DCM in general is the exacerbation of mitochondrial dysfunction [13]. We observed a significantly increased area of mitochondrial networks and a significantly greater number of junctions in *LMNA*-deficient cardiomyocytes (Fig. 1A-G). This is in line with the early stage of DCM, when an increase in mitochondrial number is observed as a compensatory mechanism to maintain energy supply [13]. A recent 3D structural study of mitochondria from patients with heart failure revealed increased mitochondrial volume and complexity, along with diverse phenotypes, including increased branching [42]. Mitochondrial hyperfusion should activate the fusion machinery, mediated by the proteins Mfn1, Mfn2, and Opa1, whereas fission, mediated by Drp1, would be suppressed [24, 43]. We performed semi-quantitative RT-PCR to assess mRNA levels of these genes (Fig. 1H-L), although their expression was unchanged between groups. We hypothesised that this could be **stress-induced mitochondrial fusion** (SIMH), which can be specifically activated, for example, through post-translational

modifications of the mentioned proteins [43], which we did not track. Further experiments should validate the mechanism behind the observed mitochondrial hyperfusion.

The observed increase in mitochondrial network may be associated with **cellular enlargement** in *LMNA*-deficient cardiomyocytes. Eccentric cell hypertrophy, or cells growing along the longitudinal axis, is one of the hallmarks of DCM [44]. We observed a significant increase in cell area in *LMNA*-deficient cardiomyocytes compared to controls (Fig. 2B), although we could not determine the direction of growth (Fig. 2C-D). Observed cellular enlargement would support a hypertrophic phenotype in *LMNA*-deficient cardiomyocytes, but further classification is needed. Of note, we also observed nuclear blebbing (Fig. 4A **DAPI**), one of the hallmarks of *LMNA*-DCM [33, 35].

A more abundant mitochondrial network may result from altered mechanobiological conditions, as disruption of the actin cytoskeleton, desmin or tubulin filaments has been reported in *LMNA*-DCM [35, 45, 46]. The cardiomyocytes with mutated *LMNA* can compensate for mitochondrial quality discrimination by increasing mitochondrial quantity. To foresee the mitochondrial quality, we focused on mitochondrial function using an indirect measure of mitochondrial potential via TMRM fluorescence. We identified a significant increase in  $\Delta\Psi_m$  in the *LMNA* group compared to controls, suggesting **mitochondrial hyperpolarisation** (Fig. 3A). Increased hyperpolarisation is associated with increased cellular respiration, as observed in the C212 cell line with the *LMNA* (G232E) mutation [47]. The hyperpolarisation was also observed in SIMH conditions and acted as a pro-survival mechanism to acute stress, such as UV irradiation [48]. Interestingly, cells undergoing SIMH exhibited increased total ATP levels due to increased oxidative phosphorylation [48]. The mitochondrial quality control system confronts not only low-potential mitochondria but also high-potential (hyperpolarised) mitochondria, which can be dangerous for the cell due to ROS overproduction in the respiratory chain. This represents a risk in the case of prolonged mitochondrial hyperpolarisation [25]. Indeed, increased basal ROS levels have been reported in several studies associated with *LMNA*-DCM [11, 49, 50]. We induced photodamage using UV irradiation in a small cellular area to induce acute oxidative stress, and we observed mitochondrial oscillations or final depolarisation (Fig. 3D). This experimental design showed in our previous study that PDM is associated with ROS increase in the damaged area, which is spread later to the mitochondrial network and finally to the cytoplasm [51]. We observed significantly improved mitochondrial survival (time to depolarisation after PDM) in *LMNA*-deficient cardiomyocytes (Fig. 3B) compared with the control group, suggesting that hyperfused mitochondria are more prone to resist acute oxidative stress. The later depolarisation time in *LMNA*-deficient cardiomyocytes was also associated with a significantly increased number of mitochondrial oscillations (Fig. 3F-G) compared to control. Similarly to our study on cardiomyoblasts [51], the mitochondrial oscillations we observed were not synchronised throughout the cell, as was described in adult primary cardiomyocytes [26]. The hyperfused mitochondria were also hyperpolarised in *LMNA*-deficient cardiomyocytes, which could represent a stress-resistant adaptive mechanism, although further validation is needed.

SIMH was associated with the unfolded protein response upon acute ER stress [27]. UPR activation was observed in patient cardiomyocytes with the *LMNA* (pS143P) mutation [18]. We observed significantly

increased levels of GP78 and CHOP, markers of ER stress, at the borderline of significance in the LMNA group (Fig. 4A-C) compared to the control, although the mRNA levels of genes involved in ER stress (*HSP5A*, *HYOU1*, *PPP1R15A*, *ATF4*) were not significantly changed (Fig. 4D-H).

iPSC-derived cardiomyocytes are well known for their relative immaturity compared to adult cardiomyocytes, although they beat and express all cardiac markers (such as cardiac troponin T) [52]. This could also explain why our observed phenotype is more adaptive and pro-survival than the expected worsened phenotype given the patient's conditions. Activation of the mitogen-activated protein kinase (MAPK) pathway preceding clinical signs or detectable molecular markers of LMNA-DCM was observed in the LMNA (H222P) mouse model [34]. Moreover, LMNA-DCM is age-dependent, associated with high penetrance of the LMNA gene in adulthood [9].

Generally, as DCM progresses, mitochondrial number declines, leading to reduced ATP and increased oxidative stress, which in turn increases  $\text{Ca}^{2+}$  uptake by mitochondria, exacerbates ROS production, and disrupts excitation-contraction coupling [13]. In the presence of another LMNA (K97E) mutation, shorter mitochondria with decreased fusion rate were observed [49]. Nevertheless, there is a lack of studies on the mitochondrial network using confocal microscopy, despite compelling evidence of mitochondrial involvement in LMNA-DCM at the functional level [12, 47, 50].

We consider the use of an age- and gender-matched commercial iPCS line, rather than an isogenic control iPCS line, as a major limitation of the study. Isogenic control is considered the gold standard for assessing the effect of a mutation, but generating such a line was not feasible for this study. To reduce the effect of this variable, we used a matched control line for gender. Our results are in line with the mechanism of LMNA-DCM, supporting that the observed phenotype is driven by the LMNA mutation.

## Conclusion

We observed changes in mitochondrial network morphology associated with the LMNA (E161K) mutation. Enlarged cardiomyocytes from the LMNA group have an abundant mitochondrial network, which is associated with their hyperpolarisation and may be activated as a stress-induced response to increased ER stress. This study supports the important role of mitochondrial (dys)function in the progression of LMNA-DCM.

## Abbreviations

mitochondrial potential ( $\Delta\Psi_m$ ); Activating transcription factor 4 (**ATF4**); binding immunoglobulin protein (**BiP**); C/EBP homologous protein (**CHOP**); Dynamin-related protein 1 (**Drp1**); endoplasmic reticulum (**ER**); glucose-regulated protein 78 (**GP78**); induced pluripotent stem cells (**iPSCs**); cardiomyocytes derived from induced pluripotent stem cells (**iPSC-CMs**); gene coding lamin A/C (**LMNA**); LMNA-deficient dilated cardiomyopathy (**LMNA-DCM**); mitofusin 1 & 2 (**Mfn1 & 2**); Optic Atrophy 1 protein (**Opa1**);

photodamage (**PDM**); reactive oxygen species (**ROS**); stress-induced mitochondrial fusion (**SIMH**); unfolded protein response (**UPR**);

## Declarations

The experiments involving both iPSC lines were performed in accordance with the Declaration of Helsinki, approved by the Ethical Committee of the Centre of Biosciences, and all cell culture procedures were in line with the national biosafety standards of Slovakia

### Data availability

The data supporting the results of our study are available from the corresponding author on request.

### Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

### AI using statement

AI (software Grammarly, Superhuman Platform Inc., USA) was used to improve human-generated texts for readability and error-free grammar, spelling and punctuation. The authors reviewed and verified the accuracy of the content.

### CRediT authorship contribution statement

**ZST:** Conceptualisation, Data Curation, Investigation, Formal analysis, Writing – original draft, Writing – review and editing; **MK:** Investigation, Formal analysis, Writing – original draft; **BJT:** Investigation, Formal analysis, Writing – Review & Editing; **AK:** Investigation, Formal analysis; **MdB:** Validation, Resources, Writing – Review & Editing; **SZ:** Validation, Resources, Writing – Review & Editing, Supervision; **KD:** Conceptualisation, Investigation, Formal analysis, Funding acquisition, Visualisation, Writing – original draft, Writing – review and editing, Project Administration, Supervision;

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## References

1. Crasto, S., My, I., & Di Pasquale, E. (2020). The Broad Spectrum of LMNA Cardiac Diseases: From Molecular Mechanisms to Clinical Phenotype. *Frontiers in Physiology*, 11. <https://doi.org/10.3389/fphys.2020.00761>
2. McNally, E. M., & Mestroni, L. (2017). Dilated Cardiomyopathy. *Circulation Research*, 121(7), 731–748. <https://doi.org/10.1161/CIRCRESAHA.116.309396>

3. Tiwari, V., Alam, M. J., Bhatia, M., Navya, M., & Banerjee, S. K. (2024). The structure and function of lamin A/C: Special focus on cardiomyopathy and therapeutic interventions. *Life Sciences*, 341, 122489. <https://doi.org/10.1016/j.lfs.2024.122489>
4. Swift, J., Ivanovska, I. L., Buxboim, A., Harada, T., Dingal, P. C. D. P., Pinter, J., ... Discher, D. E. (2013). Nuclear Lamin-A Scales with Tissue Stiffness and Enhances Matrix-Directed Differentiation. *Science*, 341(6149), 1240104. <https://doi.org/10.1126/science.1240104>
5. Brayson, D., & Shanahan, C. M. (2017). Current insights into LMNA cardiomyopathies: Existing models and missing LINC's. *Nucleus*, 8(1), 17–33. <https://doi.org/10.1080/19491034.2016.1260798>
6. Nikolova, V., Leimena, C., McMahon, A. C., Tan, J. C., Chandar, S., Jogia, D., ... Fatkin, D. (2004). Defects in nuclear structure and function promote dilated cardiomyopathy in lamin A/C-deficient mice. *The Journal of Clinical Investigation*, 113(3), 357–369. <https://doi.org/10.1172/JCI19448>
7. Arbustini, E., Pilotto, A., Repetto, A., Grasso, M., Negri, A., Diegoli, M., ... Tavazzi, L. (2002). Autosomal dominant dilated cardiomyopathy with atrioventricular block: a lamin A/C defect-related disease. *Journal of the American College of Cardiology*, 39(6), 981–990. [https://doi.org/10.1016/S0735-1097\(02\)01724-2](https://doi.org/10.1016/S0735-1097(02)01724-2)
8. Mehrabi, M., Morris, T. A., Cang, Z., Nguyen, C. H. H., Sha, Y., Asad, M. N., ... Grosberg, A. (2021). A Study of Gene Expression, Structure, and Contractility of iPSC-Derived Cardiac Myocytes from a Family with Heart Disease due to LMNA Mutation. *Annals of Biomedical Engineering*, 49(12), 3524–3539. <https://doi.org/10.1007/s10439-021-02850-8>
9. Yang, L., Sun, J., Chen, Z., Liu, L., Sun, Y., Lin, J., ... Dong, E. (2022). The LMNA p.R541C mutation causes dilated cardiomyopathy in human and mice. *International Journal of Cardiology*, 363, 149–158. <https://doi.org/10.1016/j.ijcard.2022.06.038>
10. Shah, D., Virtanen, L., Prajapati, C., Kiamehr, M., Gullmets, J., West, G., ... Aalto-Setälä, K. (2019). Modeling of LMNA-Related Dilated Cardiomyopathy Using Human Induced Pluripotent Stem Cells. *Cells*, 8(6), 594. <https://doi.org/10.3390/cells8060594>
11. Lee, J., Termglinchan, V., Diecke, S., Itzhaki, I., Lam, C. K., Garg, P., ... Wu, J. C. (2019). Activation of PDGF pathway links LMNA mutation to dilated cardiomyopathy. *Nature*, 572(7769), 335–340. <https://doi.org/10.1038/s41586-019-1406-x>
12. Qiu, H., Sun, Y., Wang, X., Gong, T., Su, J., Shen, J., ... Liang, P. (2024). Lamin A/C deficiency-mediated ROS elevation contributes to pathogenic phenotypes of dilated cardiomyopathy in iPSC model. *Nature Communications*, 15(1), 7000. <https://doi.org/10.1038/s41467-024-51318-5>
13. Ramaccini, D., Montoya-Urbe, V., Aan, F. J., Modesti, L., Potes, Y., Wieckowski, M. R., ... Matter, M. L. (2021). Mitochondrial Function and Dysfunction in Dilated Cardiomyopathy. *Frontiers in Cell and Developmental Biology*, 8. <https://doi.org/10.3389/fcell.2020.624216>
14. Sieprath, T., Corne, T. D., Nooteboom, M., Grootaert, C., Rajkovic, A., Buysschaert, B., ... De Vos, W. H. (2015). Sustained accumulation of prelamin A and depletion of lamin A/C both cause oxidative stress and mitochondrial dysfunction but induce different cell fates. *Nucleus*, 6(3), 236–246. <https://doi.org/10.1080/19491034.2015.1050568>

15. Zhou, L., Solhjoo, S., Millare, B., Plank, G., Abraham, M. R., Cortassa, S., ... O'Rourke, B. (2014). Effects of Regional Mitochondrial Depolarization on Electrical Propagation. *Circulation: Arrhythmia and Electrophysiology*, 7(1), 143–151. <https://doi.org/10.1161/CIRCEP.113.000600>
16. Carmosino, M., Gerbino, A., Schena, G., Procino, G., Miglionico, R., Forleo, C., ... Svelto, M. (2016). The expression of Lamin A mutant R321X leads to endoplasmic reticulum stress with aberrant Ca<sup>2+</sup> handling. *Journal of Cellular and Molecular Medicine*, 20(11), 2194–2207. <https://doi.org/10.1111/jcmm.12926>
17. Pietrafesa, G., De Zio, R., Scorza, S. I., Armentano, M. F., Pepe, M., Forleo, C., ... Carmosino, M. (2023). Targeting unfolded protein response reverts ER stress and ER Ca<sup>2+</sup> homeostasis in cardiomyocytes expressing the pathogenic variant of Lamin A/C R321X. *Journal of Translational Medicine*, 21(1), 340. <https://doi.org/10.1186/s12967-023-04170-y>
18. West, G., Gullmets, J., Virtanen, L., Li, S.-P., Keinänen, A., Shimi, T., ... Taimen, P. (2016). Deleterious assembly of the lamin A/C mutant p.S143P causes ER stress in familial dilated cardiomyopathy. *Journal of Cell Science*, 129(14), 2732–2743. <https://doi.org/10.1242/jcs.184150>
19. Cattelan, G., Sophie Frommelt, L., Volani, C., Colliva, A., Ciucci, G., Paldino, A., ... Rossini, A. (2023). Generation of human induced pluripotent stem cell line EURACi015-A from a patient affected by dilated cardiomyopathy carrying the Lamin A/C p.Glu161Lys mutation. *Stem Cell Research*, 71, 103172. <https://doi.org/10.1016/j.scr.2023.103172>
20. Sharma, A., Li, G., Rajarajan, K., Hamaguchi, R., Burrridge, P. W., & Wu, S. M. (2015). Derivation of highly purified cardiomyocytes from human induced pluripotent stem cells using small molecule-modulated differentiation and subsequent glucose starvation. *Journal of Visualized Experiments: JoVE*, (97), 52628. <https://doi.org/10.3791/52628>
21. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., ... Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676–682. <https://doi.org/10.1038/nmeth.2019>
22. Chaudhry, A., Shi, R., & Luciani, D. S. (2020). A pipeline for multidimensional confocal analysis of mitochondrial morphology, function, and dynamics in pancreatic  $\beta$ -cells. *American Journal of Physiology. Endocrinology and Metabolism*, 318(2), E87–E101. <https://doi.org/10.1152/ajpendo.00457.2019>
23. Artyukhov, A. S., Dashinimaev, E. B., Tsvetkov, V. O., Bolshakov, A. P., Konovalova, E. V., Kolbaev, S. N., ... Vasiliev, A. V. (2017). New genes for accurate normalization of qRT-PCR results in study of iPS and iPS-derived cells. *Gene*, 626, 234–240. <https://doi.org/10.1016/j.gene.2017.05.045>
24. Yapa, N. M. B., Lisnyak, V., Reljic, B., & Ryan, M. T. (2021). Mitochondrial dynamics in health and disease. *FEBS letters*, 595(8), 1184–1204. <https://doi.org/10.1002/1873-3468.14077>
25. Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., ... Zorov, D. B. (2018). Mitochondrial membrane potential. *Analytical biochemistry*, 552, 50–59. <https://doi.org/10.1016/j.ab.2017.07.009>

26. Aon, M. A., Cortassa, S., & O'Rourke, B. (2008). Mitochondrial Oscillations in Physiology and Pathophysiology. *Advances in experimental medicine and biology*, 641, 98–117.  
[https://doi.org/10.1007/978-0-387-09794-7\\_8](https://doi.org/10.1007/978-0-387-09794-7_8)
27. Lebeau, J., Saunders, J. M., Moraes, V. W. R., Madhavan, A., Madrazo, N., Anthony, M. C., & Wiseman, R. L. (2018). The PERK Arm of the Unfolded Protein Response Regulates Mitochondrial Morphology during Acute Endoplasmic Reticulum Stress. *Cell Reports*, 22(11), 2827–2836.  
<https://doi.org/10.1016/j.celrep.2018.02.055>
28. Wei, S., Zhang, N., Zhang, H., Chen, Z., Li, S., Wu, W., ... Cheng, Q. (2026). Endoplasmic reticulum stress in disease pathogenesis: its implications for therapy. *Signal Transduction and Targeted Therapy*, 11(1), 136. <https://doi.org/10.1038/s41392-026-02600-z>
29. Yang, Y., Liu, L., Naik, I., Braunstein, Z., Zhong, J., & Ren, B. (2017). Transcription Factor C/EBP Homologous Protein in Health and Diseases. *Frontiers in Immunology*, 8, 1612.  
<https://doi.org/10.3389/fimmu.2017.01612>
30. Hamilton, S., & Terentyev, D. (2022). ER stress and calcium-dependent arrhythmias. *Frontiers in Physiology*, 13. <https://doi.org/10.3389/fphys.2022.1041940>
31. Kojima, E., Takeuchi, A., Haneda, M., Yagi, F., Hasegawa, T., Yamaki, K.-I., ... Isobe, K.-I. (2003). The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress—elucidation by GADD34-deficient mice. *The FASEB Journal*, 17(11), 1–18.  
<https://doi.org/10.1096/fj.02-1184fje>
32. Verdonschot, J. A. J., Hazebroek, M. R., Ware, J. S., Prasad, S. K., & Heymans, S. R. B. (2019). Role of Targeted Therapy in Dilated Cardiomyopathy: The Challenging Road Toward a Personalized Approach. *Journal of the American Heart Association*, 8(11), e012514.  
<https://doi.org/10.1161/JAHA.119.012514>
33. Neupane, B., Pradhan, K., Ortega-Ramirez, A. M., Aidery, P., Kucikas, V., Marks, M., ... Gramlich, M. (2022). Personalized Medicine Approach in a DCM Patient with LMNA Mutation Reveals Dysregulation of mTOR Signaling. *Journal of Personalized Medicine*, 12(7), 1149.  
<https://doi.org/10.3390/jpm12071149>
34. Muchir, A., Pavlidis, P., Decostre, V., Herron, A. J., Arimura, T., Bonne, G., & Worman, H. J. (2007). Activation of MAPK pathways links LMNA mutations to cardiomyopathy in Emery-Dreifuss muscular dystrophy. *The Journal of Clinical Investigation*, 117(5), 1282–1293.  
<https://doi.org/10.1172/JCI29042>
35. Laurini, E., Martinelli, V., Lanzicher, T., Puzzi, L., Borin, D., Chen, S. N., ... Pricl, S. (2018). Biomechanical defects and rescue of cardiomyocytes expressing pathologic nuclear lamins. *Cardiovascular Research*, 114(6), 846–857. <https://doi.org/10.1093/cvr/cvy040>
36. Garcia-Pavia, P., Palomares, J. F. R., Sinagra, G., Barriales-Villa, R., Lakdawala, N. K., Gottlieb, R. L., ... REALM-DCM Investigators. (2024). REALM-DCM: A Phase 3, Multinational, Randomized, Placebo-Controlled Trial of ARRY-371797 in Patients With Symptomatic LMNA-Related Dilated

- Cardiomyopathy. *Circulation. Heart Failure*, 17(7), e011548.  
<https://doi.org/10.1161/CIRCHEARTFAILURE.123.011548>
37. Sébillon, P., Bouchier, C., Bidot, L. D., Bonne, G., Ahamed, K., Charron, P., ... Komajda, M. (2003). Expanding the phenotype of LMNA mutations in dilated cardiomyopathy and functional consequences of these mutations. *Journal of Medical Genetics*, 40(8), 560–567.  
<https://doi.org/10.1136/jmg.40.8.560>
  38. Perrot, A., Hussein, S., Ruppert, V., Schmidt, H. H. J., Wehnert, M. S., Duong, N. T., ... Özcelik, C. (2009). Identification of mutational hot spots in LMNA encoding lamin A/C in patients with familial dilated cardiomyopathy. *Basic Research in Cardiology*, 104(1), 90–99.  
<https://doi.org/10.1007/s00395-008-0748-6>
  39. Mewborn, S. K., Puckelwartz, M. J., Abuisneineh, F., Fahrenbach, J. P., Zhang, Y., MacLeod, H., ... McNally, E. (2010). Altered Chromosomal Positioning, Compaction, and Gene Expression with a Lamin A/C Gene Mutation. *PLOS ONE*, 5(12), e14342. <https://doi.org/10.1371/journal.pone.0014342>
  40. Bhattacharjee, P., Dasgupta, D., & Sengupta, K. (2017). DCM associated LMNA mutations cause distortions in lamina structure and assembly. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1861(11, Part A), 2598–2608. <https://doi.org/10.1016/j.bbagen.2017.08.016>
  41. Borin, D., Peña, B., Chen, S. N., Long, C. S., Taylor, M. R. G., Mestroni, L., & Sbaizero, O. (2020). Altered microtubule structure, hemichannel localization and beating activity in cardiomyocytes expressing pathologic nuclear lamin A/C. *Heliyon*, 6(1). <https://doi.org/10.1016/j.heliyon.2020.e03175>
  42. Vue, Z., Ajayi, P. T., Neikirk, K., Murphy, A. C., Prasad, P., Jenkins, B. C., ... Hinton, A. (2023, December 14). Human Heart Failure Alters Mitochondria and Fiber 3D Structure Triggering Metabolic Shifts. *bioRxiv*. <https://doi.org/10.1101/2023.11.28.569095>
  43. Shutt, T. E., & McBride, H. M. (2013). Staying cool in difficult times: mitochondrial dynamics, quality control and the stress response. *Biochimica Et Biophysica Acta*, 1833(2), 417–424.  
<https://doi.org/10.1016/j.bbamcr.2012.05.024>
  44. Nakamura, M., & Sadoshima, J. (2018). Mechanisms of physiological and pathological cardiac hypertrophy. *Nature Reviews Cardiology*, 15(7), 387–407. <https://doi.org/10.1038/s41569-018-0007-y>
  45. Macquart, C., Jüttner, R., Morales Rodriguez, B., Le Dour, C., Lefebvre, F., Chatzifrangkeskou, M., ... Muchir, A. (2019). Microtubule cytoskeleton regulates Connexin 43 localization and cardiac conduction in cardiomyopathy caused by mutation in A-type lamins gene. *Human Molecular Genetics*, 28(24), 4043–4052. <https://doi.org/10.1093/hmg/ddy227>
  46. Mounkes, L. C., Kozlov, S. V., Rottman, J. N., & Stewart, C. L. (2005). Expression of an LMNA-N195K variant of A-type lamins results in cardiac conduction defects and death in mice. *Human Molecular Genetics*, 14(15), 2167–2180. <https://doi.org/10.1093/hmg/ddi221>
  47. Ignatieva, E. V., Ivanova, O. A., Komarova, M. Y., Khromova, N. V., Polev, D. E., Kostareva, A. A., ... Dmitrieva, R. I. (2020). LMNA Mutations G232E and R482L Cause Dysregulation of Skeletal Muscle

- Differentiation, Bioenergetics, and Metabolic Gene Expression Profile. *Genes*, 11(9), 1057. <https://doi.org/10.3390/genes11091057>
48. Tondera, D., Grandemange, S., Jourdain, A., Karbowski, M., Mattenberger, Y., Herzig, S., ... Martinou, J. (2009). SLP-2 is required for stress-induced mitochondrial hyperfusion. *The EMBO Journal*, 28(11), 1589–1600. <https://doi.org/10.1038/emboj.2009.89>
49. Nath, S., Prusty, D., Islam, S. R., Manna, S. K., & Sengupta, K. (2026). Attenuated lamin A-prohibitin2 interaction leads to mitochondrial dysfunction in LMNA 289 A>G-mediated dilated cardiomyopathy. *The Journal of Biological Chemistry*, 302(4), 111274. <https://doi.org/10.1016/j.jbc.2026.111274>
50. Wong, L. Y., Torfs, T., Vanherle, S. J. V., Janssen, J., Claes, G. R. F., Stroeks, S. L. V. M., ... Nabben, M. (2025). Clinical and metabolic consequences of a historic pathogenic lamin A/C founder variant. *Scientific Reports*, 15(1), 23842. <https://doi.org/10.1038/s41598-025-08495-0>
51. Kurekova, S., Sevcikova Tomaskova, Z., Andelova, N., Macejova, D., Cervienkova, M., Brtko, J., ... Mackova, K. (2022). The effect of all-trans retinoic acid on the mitochondrial function and survival of cardiomyoblasts exposed to local photodamage. *Cell Biology International*, 46(6), 947–964. <https://doi.org/10.1002/cbin.11784>
52. Scuderi, G. J., & Butcher, J. (2017). Naturally Engineered Maturation of Cardiomyocytes. *Frontiers in Cell and Developmental Biology*, 5. <https://doi.org/10.3389/fcell.2017.00050>

## Figures

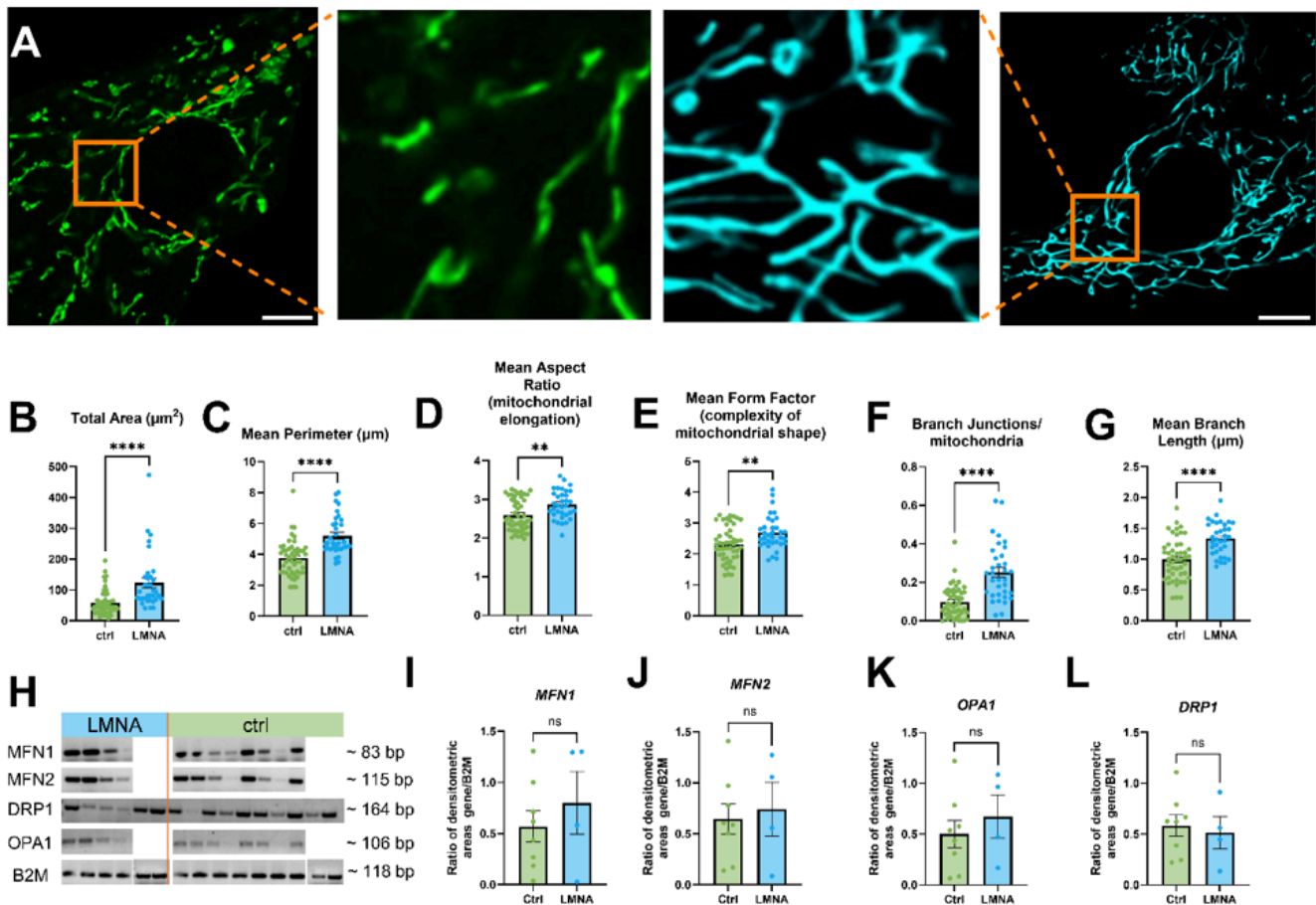


**Figure 1**

**Mitochondrial networks in *LMNA*-deficient and control iPSC-derived cardiomyocytes.** **A:** Representative fluorescent images of mitochondrial networks from control cardiomyocyte (SCT1 line, left, in green) and *LMNA*-deficient cardiomyocyte (EURACi015-A line, right, in bright blue/cyan). Orange squares indicate the selected zoomed regions in the middle. Scale is 10  $\mu$ m. **B:** Total area of mitochondrial networks, **C:** Mean perimeter of mitochondria, **D:** Mean aspect ratio interpreted as mitochondrial elongation, **E:** Mean Form Factor interpreted as complexity of mitochondrial shape, **F:** Branch junction/mitochondria ratio, **G:** Mean branch length between control and *LMNA*-deficient cardiomyocytes. Data are visualised as individual data points, and bar graphs depict mean values with SEM (n(control) = 48, n(LMNA) = 34). \*\*: ~0.003, \*\*\*\*: <0.0001. **H:** Gene expression of the selected genes involved in mitochondrial dynamics (*MFN1*, *MFN2*, *DRP1*, *OPA1*) and *B2M* as a housekeeper. The gels for selected genes are shown with base-pair sizes. Samples from the LMNA and control groups are separated by the orange line. **I:** Comparison of the ratio of the densitometric areas of the *MFN1* gene to the *B2M* gene. **J:** Comparison of the ratio of the densitometric areas of the *MFN2* gene to the *B2M* gene. **K:** Comparison of the ratio of the densitometric areas of the *OPA1* gene to the *B2M* gene. **L:** Comparison of the ratio of the densitometric areas of the *DRP1* gene to the *B2M* gene. Data are visualised as individual data points, bar graphs depict mean values with SEM (n(control) = 8/10, n(LMNA) = 4/6). ns: non-significant.

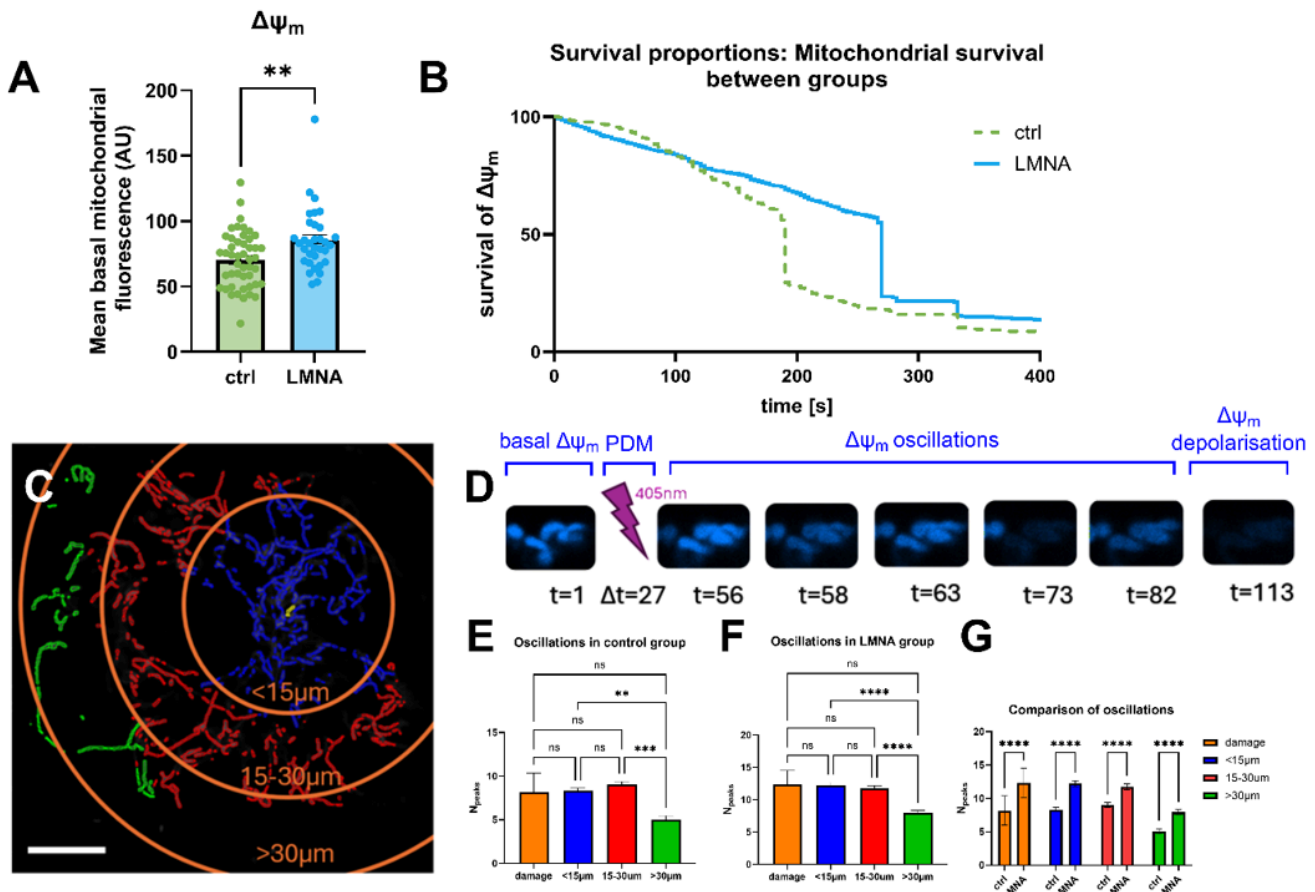
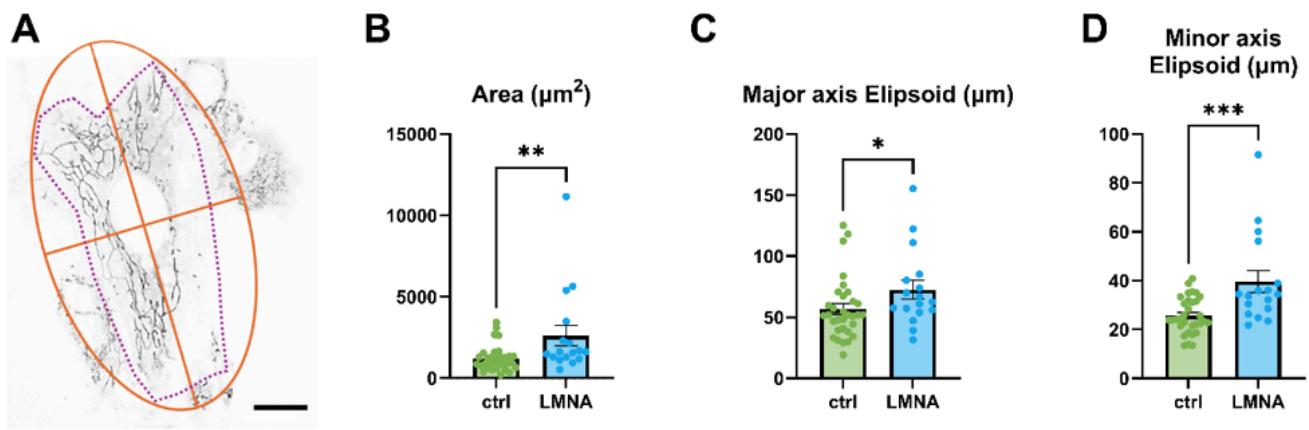
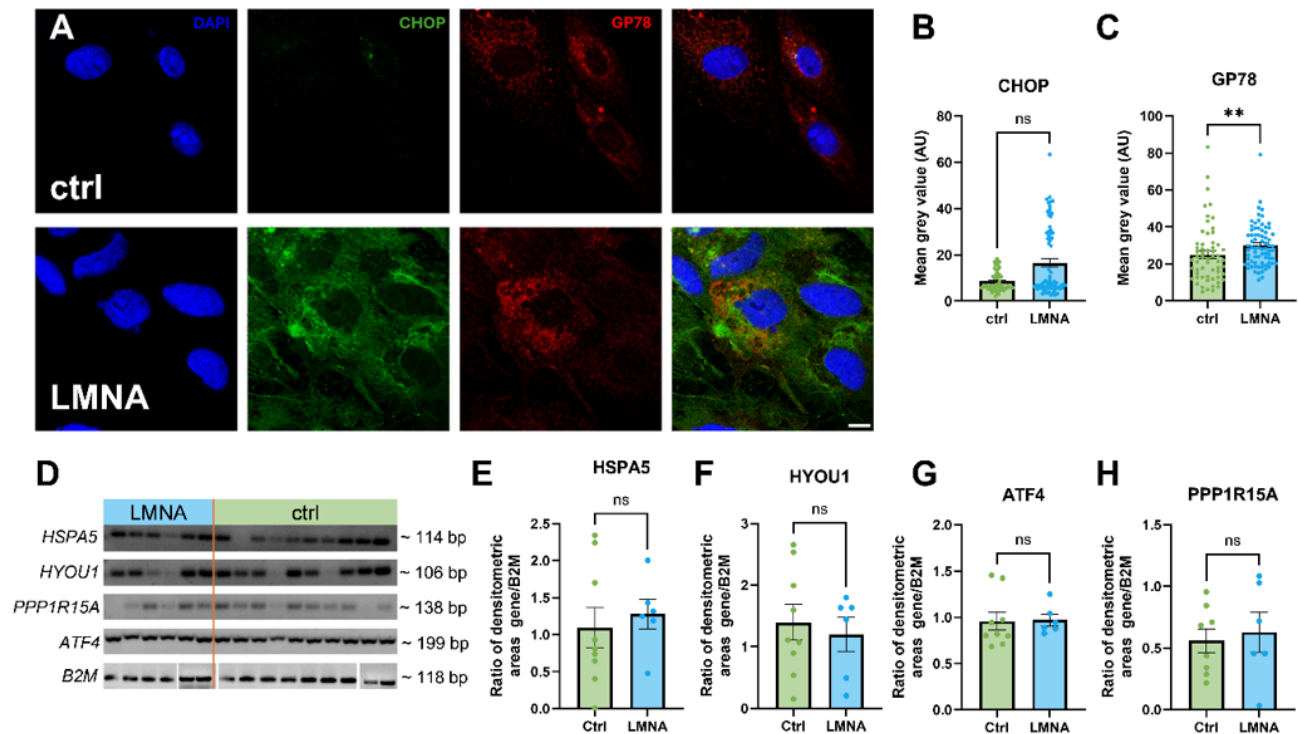


**Figure 3**

**Changed mitochondrial potential ( $\Delta\Psi_m$ ) in *LMNA*-deficient cardiomyocytes. **A:** Mean fluorescence of TMRM signal (AU) at basal condition between control and *LMNA*-deficient cardiomyocytes. Data are visualised as individual data points, and bar graphs depict mean values with SEM ( $n(\text{control}) = 47$ ,  $n(\text{LMNA}) = 33$ ). **B:** Survival analysis of mitochondrial depolarisation events between groups. **C:** TMRM-labelled *LMNA*-deficient cardiomyocytes overlayed with ROI mask of all mitochondria grouped by the distance from the PDM area (yellow). Less than 15  $\mu\text{m}$ : deep blue, 15 – 30  $\mu\text{m}$ : red, more than 30  $\mu\text{m}$ : green. Scale is 10  $\mu\text{m}$ . **D:** Time-dependent change of TMRM signal reflects the change in  $\Delta\Psi_m$ . At  $t=1\text{s}$ , there is a basal  $\Delta\Psi_m$  that oscillates after photodamage (PDM) over time (56 – 82s). The  $\Delta\Psi_m$  depolarisation is the final stage when mitochondria are no longer able to produce ATP. **E:** Number of oscillation peaks ( $N_{\text{peaks}}$ ) per mitochondria in the control group in the damaged zone, the nearest zone to PDM ( $<15\text{ }\mu\text{m}$ ), the middle zone (15-30  $\mu\text{m}$ ), and the far zone ( $>30\text{ }\mu\text{m}$ ). **F:** Number of oscillation peaks ( $N_{\text{peaks}}$ ) per mitochondria in the *LMNA* group in the damaged zone, the nearest zone to PDM ( $<15\text{ }\mu\text{m}$ ), the middle zone (15-30  $\mu\text{m}$ ), and the far zone ( $>30\text{ }\mu\text{m}$ ). **G:** Comparison of the numbers of oscillation peaks ( $N_{\text{peaks}}$ ) per mitochondria between the control and the *LMNA* group in the damaged zone, the nearest zone to PDM ( $<15\text{ }\mu\text{m}$ ), the middle zone (15-30  $\mu\text{m}$ ), and the far zone ( $>30\text{ }\mu\text{m}$ ). Data are visualised as bar graphs depicting mean values with SEM ( $n(\text{damage/control}) = 5$ ,  $n(<15\text{ }\mu\text{m/control}) = 221$ ,  $n(15-30\text{ }\mu\text{m/control}) = 169$ ,  $n(>30\text{ }\mu\text{m/control}) = 23$ ,  $n$  of mitochondria is subsequent when at least 3 differentiations were used:  $n(\text{damage/LMNA}) = 6$ ,  $n(<15\text{ }\mu\text{m/LMNA}) = 304$ ,  $n(15-30\text{ }\mu\text{m/LMNA}) = 333$ ,  $n(>30\text{ }\mu\text{m/LMNA}) = 200$ . ns: non-significant, \*\*:  $\sim 0.006$ , \*\*\*:  $\sim 0.0005$ , \*\*\*\*:  $< 0.0001$ .**



## Figure 4

**Increased ER stress in LMNA-deficient cardiomyocytes. A:** Immunofluorescent images of control (upper row) and LMNA-deficient cardiomyocyte (lower row) stained for DAPI (blue), CHOP- Alexa 488 (green), GP78 – Texas Red 594 and overlay of the selected channels. Scale is 10  $\mu$ m. **B:** Comparison of mean grey values (AU) of fluorescent CHOP protein signal from control and LMNA group. **C:** Comparison of mean grey values (AU) of fluorescent GP78 protein signal from control and LMNA group. Data are visualised as individual data points, bar graphs depict mean values with SEM (n(control) = 59, n(LMNA) = 74). ns: non-significant, \*\*: ~0.003. **D:** Gene expression of the selected ER stress genes (*HSP5A*, *HYOU1*, *PPP1R15A*, *ATF4*) and *B2M* as a housekeeper. The gels for selected genes are shown with base-pair sizes. Samples from the LMNA and control groups are separated by the orange line. **E:** Comparison of the ratio of the densitometric areas of the *HSPA5* gene to the *B2M* gene. **F:** Comparison of the ratio of the densitometric areas of the *HYOU1* gene to the *B2M* gene. **G:** Comparison of the ratio of the densitometric areas of the *ATF4* gene to the *B2M* gene. **H:** Comparison of the ratio of the densitometric areas of the *PPP1R15A* gene to the *B2M* gene. Data are visualised as individual data points, bar graphs depict mean values with SEM (n(control) = 9, n(LMNA) = 6). ns: non-significant.