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Melusin protects from cardiac rupture and improves functional remodelling after myocardial infarction.

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15 September 2026

SUPPLEMENTARY MATERIAL

"Melusin protects from cardiac rupture and improves functional remodelling in mice after myocardial infarction"

Bernhard Unsöld et al.

Matherial and Methods

Induction of Myocardial infarction

Mice weighing 20±2 g (females) and 25±2 g (males) aged 7-8 weeks were anaesthetised by isoflurane inhalation and endotracheally intubated. Anaesthesia was maintained by continuous inhalation of 1.5 % isoflurane in room air. They were placed in a right lateral decubitus position and the fur was removed from the left hemithorax by use of depilatory cream. A left lateral thoracotomy was performed to expose the heart. Following pericardiotomy the LAD was visualized and ligated with a 9-0 prolene suture with an atraumatic needle. Successful ligation was confirmed by bleaching and hypokinesis of the perfused myocardium. Following expansion of the lungs by compression of the exhalation tube the thorax and the skin were closed with separate single 6-0 prolene sutures. Isoflurane inhalation was stopped and the mice were ventilated until they breathed spontaneously. They were kept separate on a warming plate until fully awake. Postoperative analgesia was provided by metamizole added to the drinking water (1.33 mg/ml) for 7 days. The sham operation was performed similarly however no coronary artery ligation was performed.

Determination of myocardium at risk

The volume of myocardium at risk was determined in a subset of animals by retrograde coronary perfusion with Evan's blue (1%) 3-4 hours after coronary ligation. The perfused myocardium stained by Evan's blue was dissected from the non stained ischemic myocardium under a microscope and the weight of both portions was determined.

Measurement of infarct size

Paraffin-embedded tissues of animals sacrificed 3 days after surgery were cut in 5 µm sections beginning 900 µm from the apex of each heart to be safely within the infracted tissue. Haematoxylin/eosin stained tissues were used for the calculation of the infarct size of the heart using the area measurement method[1]. Infarct size was defined as the sum of the epicardial and endocardial infarct circumference divided by the sum of the total LV epicardial and endocardial circumferences using computer based planimetry. Quantitative assessments were performed with image analysis software AxioVision Rel.4.7 (Carl Zeiss).

Measurements of cell contraction and Ca^{2+} transients 3 days after MI

As these measurements were performed at Leuven/Belgium these 24 mice were the only ones not operated at the central site in Goettingen. However the operations were performed by one operator according to the standard operational procedure established in Goettingen. Mice were sacrificed 72 hours after LAD ligation and single cardiac myocytes were isolated as described previously[2]. Briefly, mice received an IP injection of pentobarbital and heparin and hearts were quickly removed through a thoracotomy under full anaesthesia. The aorta was cannulated on a Langendorff setup for retrograde perfusion with an enzyme solution (collagenase B, Roche, and protease XIV, Sigma). After digestion, single cells were dispersed from the viable LV myocardium (RV, MI scar and the border zone were discarded). Measurements of contraction and intracellular calcium concentration $[Ca^{2+}]_i$ were performed as described previously[2]. Briefly, cell shortening was measured using edge tracking (Ionoptix) during electrical field stimulation at 1 Hz; action potentials and $[Ca^{2+}]_i$ were measured during whole-cell patch clamp with fluo-3 as indicator loaded via the patch pipette. During field stimulation and patch-clamp experiments cells were superfused with normal Tyrode solution (in mmol/l: 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, 11.8 HEPES and 10 glucose, pH was adjusted to 7.40 with NaOH). The pipette solution for whole-cell patch clamp contained (in mmol/l): 120 K-aspartate, 20 KCl, 10 NaCl, 10 K-HEPES, 5 MgATP, and 0.05 K_5-fluo-3, pH 7.2. All experiments were performed at 35^{o}C.

Measurements of cell contraction and Ca^{2+} transients 14 days after MI

Shortening and Ca^{2+} measurements were performed using an IonOptix fluorescence and Myocam setup (IonOptix, Milton/USA) mounted to a Nikon Eclipse TE300 microscope as described previously[3]. Single cardiac myocytes were isolated using digestion with Liberase Blendzyme (Roche) and were loaded with the acetoxymethyl ester form of Fluo-3 (10µmol/l, Molecular Probes, Eugene/USA) by incubation for 15 minutes at room temperature. Emitted fluorescence was collected using a photomultiplier at ≈535±20nm and Ca^{2+} transient amplitudes were estimated by calculating peak fluorescence divided through baseline fluorescence (F/F_0) after subtracting background fluorescence. Myocyte shortening was assessed by measuring sarcomere length and percentual myocyte fractional shortening was calculated from this measurement.

Immunohistochemistry for CD18, CD31 and WGA staining

Histochemical analyses were performed on 5 µm-thick frozen cross sections through the left ventricle. For each mouse, 4 sections, approximately 500 µm apart, were analyzed and findings averaged. For immunohistochemical analyses rat monoclonal antibody against CD18 (BMA, Augst, Switzerland) was used according to manufacturer instructions. Biotinylated

rabbit anti-rat IgG (Dako Cytomation, Milano, Italy) was used as the secondary antibody. Immunoreactivity was detected with the streptABCComplex/HRP system (DakoCytomation; Milano, Italy) and developed with DAB (methanol 3,3 diamino-benzide, Roche Diagnostic Corp., Milano, Italy). Monoclonal rat anti-mouse antibodies against CD31 were used to detect endothelial cells (sc-18916; dilution, 1:40; SantaCruz Biotechnology). After incubation overnight at 4°C and subsequent washing in phosphate-buffered saline (PBS), phycoerythrin (PE)-labeled secondary goat anti-rat antibody (dilution, 1:400; Molecular Probes) was added, followed by incubation with fluorescein (FITC)-labeled wheat germ agglutinin (WGA; dilution, 1:200; Molecular Probes) in order to delineate cardiomyocytes and 4',6-diamidino-2-phenylindole (DAPI; dilution, 1:500; Sigma) to visualize cell nuclei. Sections were mounted in VectaShield (Vector) and inspected on a Zeiss axiovert fluorescence microscope. The number of CD31-positive endothelial cells was evaluated on 4 randomly selected microscope fields (400x magnification) per section and expressed per mm^2 or cardiomyocyte fiber. The cardiomyocyte cross sectional area (CSA) was determined after WGA staining using specialized image analysis software (Image ProPlus).

Phospho-ERK1/2 immunohistochemistry

Tissue sections (4 µm) were cut from formalin fixed, paraffin embedded hearts from wild type and melusin transgenic mice 3 days after MI. For p-ERK1/2 staining (antibody from Cell Signaling) manufacturer's instructions were followed. Briefly, sections were de-waxed and rehydrated and antigen was retrieved incubating the glass slides in sodium citrate for 10 minutes at sub boiling temperature in a microwave oven. Sections were incubated with anti-p-ERK1/2 antibodies (1:100) overnight at 4°C. Biotinylated secondary anti-rabbit antibodies (1:200) were left on the sections for 30 min at RT. Immunostaining was performed using avidin-biotin-HRP system with diaminobenzidine as chromogen. Sections were not counterstained. For the analysis of p-ERK1/2 positive nuclei, pictures were taken at 400X magnification, using the same light intensity, from specific regions of the sections, as indicated in the results. Images were analysed using Image J software, without any brightness/contrast manipulation. To count the number of p-ERK positive nuclei, a comparable threshold level was set for all images analysed, to highlight only stained nuclei. Using the *Analyze Particles* tool, the highlighted nuclei were counted, excluding particles with an area outside the size range of cardiomyocyte nuclei (to exclude non-cardiomyocyte nuclei and non-nuclei spots from the count).

Detection of Apoptosis

Apoptotic cells were detected by TUNEL using the In Situ Detection Kit, Fluorescein (Roche, Almere, Netherlands) according to the manufacturer's instructions. Briefly, paraffin sections (5 µm) were pre-treated with Proteinase K (20 µg/ml in PBS, Roche) for 15 min at RT.

Subsequently, sections were incubated with 3% BSA and 20% fetal bovine serum in PBS (30 min, RT) to reduce background. Sections were incubated in a dark, humidified chamber with the TUNEL reaction mixture (60 min, 37°C). After washing (3x with PBS) sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, U.S.). Sections were analysed by fluorescent microscopy (Leica DM5000B; Leica Microsystems, Rijswijk, Netherlands) using the Leica Qwin software program. Apoptosis was quantified by analysing 3 pictures of the non-infarcted septum on 2 sections per heart, generating a median value for each animal. Data are expressed as the percentage of TUNEL-positive cells over DAPI stained nuclei for each group.

Sirius Red staining

Sirius Red staining of paraffin embedded cross sections was used for fibrosis calculation. Ten microscopic pictures in 10-fold magnification were taken from one slide of each animal and analysed by the image-processing software ImageJ (shareware from the NIH). After manually setting a threshold using a randomly chosen subset of the pictures, we measured the relationship of Sirius red-stained area (connective tissue in red) to total area of the picture and calculated the percentage of connective tissue.

RNA isolation and real-time quantitative PCR for cytokines and growth factors 3 days after MI

RNA was extracted from frozen heart tissue using Trizol (Invitrogen). 1 µg of total RNA was retro-transcribed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). Singleplex TaqMan real time PCR was performed using the Roche Universal Probe Library system in combination with specific primers indicated in supplementary Table 1. 18S rRNA expression was measured to normalize gene expression using TaqMan Applied Biosystems Eukaryotic 18S rRNA Endogenous Control assay. PCRs were run on an Applied Biosystems 7900HT system and results were analyzed with the provided software.

RNA isolation and real-time quantitative PCR for metabolic enzymes 2 weeks after MI

Total RNA was isolated with TRI reagent (Sigma, St Louis, MO, USA) according to the manufacturer's protocol and complemented with an additional wash step of 70% ethanol to increase the purity of the RNA. Integrity of the RNA was checked by means of the 260/280 nm ratio. 250 ng of RNA was treated with DNase type I (Sigma) and subsequently used for cDNA synthesis (Iscript cDNA synthesis kit, Bio-Rad Inc., Hercules, CA, USA). QPCR assays were performed on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) using the iQ SYBR-Green supermix (Biorad). qPCR was performed for the nuclear peroxisome

proliferator-activated receptor alpha (*Pparα*), its co-activator protein peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1α*) as well as for the long-chain-fatty-acid-CoA ligase 1 (*Acsl1*) and the mitochondrial trifunctional enzyme subunit alpha (*Hadha*), two enzymes involved in fatty acid metabolism. Primer sequences are given in supplementary Table 1. For all primer sets, temperature and dilution curves were assessed to check for linearity. Expression levels were normalized to the housekeeping gene cyclophilin A (*Ppia*). Relative changes in expression were calculated using GeneX software (Bio-Rad). The values were normalized for the value of sham operated female wild type mice for which the group average was arbitrarily set to 1.

RNA isolation and real-time quantitative PCR for ANF

For the measurement of *Nppa* quantitative real-time RT-PCR was performed using the standard curve method with the Taqman instrument (ABI7000) and SYBR green. Experiments were performed in duplicates. mRNA content was normalized to the mRNA content of *Gapdh* in the same sample. Primers sequences are given in supplementary Table 1.

Western blotting

Calcium handling proteins

Western blotting was performed as described previously[4]. In brief, protein content in the samples was determined and similar amounts of total protein per sample were loaded. Primary antibodies against calsequestrin (Dianova, Hamburg, Germany), total phospholamban (PLB), Thr^{17}-phosphorylated PLB and Ser^{16}-phosphorylated PLB (Badrilla, Leeds, UK), troponin-I (Tn-I, Chemicon, Dundee, Scotland), $Ser^{22/23}$-phosphorylated Tn-I (Cell Signaling Technology, Boston, MA, USA), SR-Ca^{2+}-ATPase (SERCA2a, Santa Cruz, California, USA). Antibody binding was detected by horseradish peroxidase-conjugated secondary antibodies. The values were normalized for the value of sham operated female wild type mice for which the group average was arbitrarily set to 1.

Melusin and HSP70

Frozen heart extracts were reconstituted in cold lysis buffer containing phosphatase and protease inhibitors (composition in mmol/l: imidazole, 50, pH 7.0; KCl, 300; NaF, 10; EGTA, 1; MgCl2, 0.5; β-glycerophosphate, 10; NaVO4, 1; dithiothreitol, 1; phenylmethylsulfonyl fluoride, 0.1; and benzamidine, 1) and homogenized by sonication on ice. Equal amounts of denatured proteins were separated by 10-8 % acrylamide gel electrophoresis, transferred to nitrocellulose or polyvinylidene difluoride membranes, blocked overnight in 0.1 % Tris-buffered saline/Tween-20 buffer containing 5 % (wt/vol) nonfat dry milk (Fluka) or albumin

according to the manufacturer's protocol and analyzed with primary antibodies to melusin and HSP70. Vinculin was used as loading control).

Vinculin antibody was from Sigma, St. Louis, MO, USA and HSP70 antibody was from Stressgen, Victoria, British Columbia, Canada. The antibody used to detect melusin was custom made[5]. Horseradish peroxidase-conjugated secondary antibodies were used according to the manufacturer's instruction. Protein bands were visualized using ECL reagent (Perkin Elmer, Boston, USA) and detected using Kodak film or Geliance 600, Perkin Elmer. Band intensity was quantified by densitometry of immunoblots using ImageJ program. Results of densitometric analyses were expressed as ratios to loading control.

SUPPLEMENTARY TABLE 1

Gene	**Forward primer**	**Reverse primer**
Acsl1	GAGGGTGAGGTGTGTGTGAA	CAGCTGTTCTTGCTGGGTCT
Acta2	CCCACCCAGAGTGGAGAA	ACATAGCTGGAGCAGCGTCT
Ccl2	AACTCTCACTGAAGCCAGCTCT	GTGGGGCGTTAACTGCAT
Ccl3	AGATTCCACGCCAATTCATC	GCCGGTTTCTCTTAGTCAGGA
Col1α1	CATGTTCAGCTTTGTGGACCT	GCAGCTGACTTCAGGGATGT
Cxcl10	GCTGCCGTCATTTTCTGC	TCTCACTGGCCCGTCATC
Cxcl2	AAAATCATCCAAAAGATACTGAACAA	CTTTGGTTCTTCCGTTGAGG
Fn1	CGGAGAGAGTGCCCCTACTA	CGATATTGGTGAATCGCAGA
Gapdh	ATGGTGAAGGTCGGTGTGA	AATCTCCACTTTGCCACTGC
Gdf15	GAGCTACGGGGTCGCTTC	GGGACCCCAATCTCACCT
Hadha	GGTGTCCCTGAAGTGTTGCT	TCTGTCTGCACGAATGTTCC

Igf1	CAAAAGCAGCCCGCTCTA	TCGATAGGGACGGGGACT
Il10	GCTCCTAGAGCTGCGGACT	TGTTGTCCAGCTGGTCCTTT
Il18	CAAACCTTCCAAATCACTTCCT	TCCTTGAAGTTGACGCAAGA
Il1b	AGTTGACGGACCCCAAAAG	GAAGCTGGATGCTCTCATCA
Il6	GCTACCAAACTGGATATAATCAGGA	CCAGGTAGCTATGGTACTCCAGAA
Itgam	CAATAGCCAGCCTCAGTGC	GAGCCCAGGGGAGAAGTG
Klf5	CCGGAGACGATCTGAAACAC	CAGATACTTCTCCATTTCACATCTTG
Marco	GGCACCAAGGGAGACAAA	TCCCTTCATGCCCATGTC
Myh6	CGCATCAAGGAGCTCACC	CCTGCAGCCGCATTAAGT
Myh7	CGCATCAAGGAGCTCACC	CTGCAGCCGCAGTAGGTT
Nppa (2 weeks)	CCTGTGTACAGTGCGGTGTC	CCTCATCTTCTACCGGCATC
Pgf	CTGGGTTGGCTGTGCATT	GGCACCACTTCCACTTCTGT
Pparα	TATTCGGCTGAAGCTGGTGTAC	CTGGCATTTGTTCCGGTTCT
Ppargc1α	TTGAGGAATGCACCGTAAATC	GCTCGAAGTCAGTTTCATTCG
Retnla	CCCTCCACTGTAACGAAGACTC	CACACCCAGTAGCAGTCATCC
Tgfb1	TGGAGCAACATGTGGAACTC	CAGCAGCCGGTTACCAAG
Tmsb4x	CACACATAAAGCGGCGTTC	GACATGGTTGCTGGAAGGAG
Tnf	CTGTAGCCCACGTCGTAGC	TTGAGATCCATGCCGTTG

Gene	full name
Acsl1	Long-chain-fatty-acid—CoA ligase 1
Acta2	actin, alpha 2, smooth muscle, aorta
Ccl2	chemokine (C-C motif) ligand 2
Ccl3	chemokine (C-C motif) ligand 3
Col1α1	collagen, type I, alpha 1
Cxcl10	chemokine (C-X-C motif) ligand 10
Cxcl2	chemokine (C-X-C motif) ligand 2
Eno1	enolase 1
Eno3	enolase 3
Fn1	fibronectin 1
Gdf15	growth differentiation factor 15
Glut1	glucose transporter member 1
Glut4	glucose transporter member 4
Hadha	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase
Ifnγ	interferon gamma
Igf1	insulin-like growth factor 1
Il10	interleukin 10
Il18	interleukin 18
Il1β	interleukin 1 beta
Il6	interleukin 6
Itgam	integrin alpha M
Klf5	Kruppel-like factor 5
Marco	macrophage receptor with collagenous structure
Mmp9	matrix metallopeptidase 9
Myh6	myosin, heavy polypeptide 6, cardiac muscle, alpha
Myh7	Myh7 and Name: myosin, heavy polypeptide 7, cardiac muscle, beta
Pgf	placental growth factor
Ppargc1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pparα	peroxisome proliferator-activated receptor alpha
Pparδ	peroxisome proliferator-activated receptor delta
Pparγ	peroxisome proliferator-activated receptor gamma
Retnla	resistin like alpha
Tgfβ1	transforming growth factor, beta 1
Tmsb4x	thymosin, beta 4, X chromosome

Tnf	tumor necrosis factor
Vegfα	vascular endothelial growth factor A

Supplementary References

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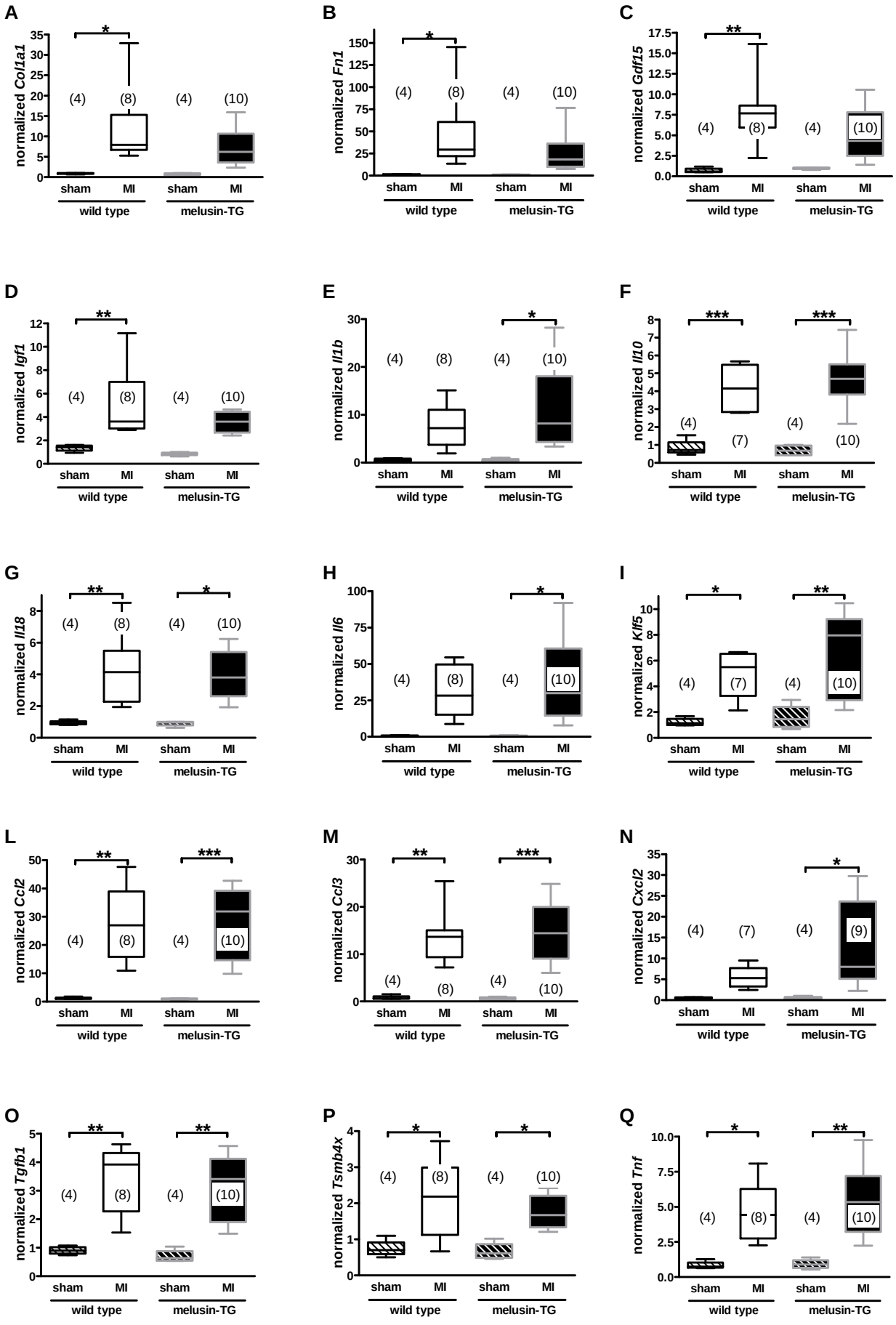
Figure S1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normalized Il18
H
normalized Il6
I
normalized Klf5
L
normalized Ccl2
M
normalized Ccl3
N
normalized Cxcl2
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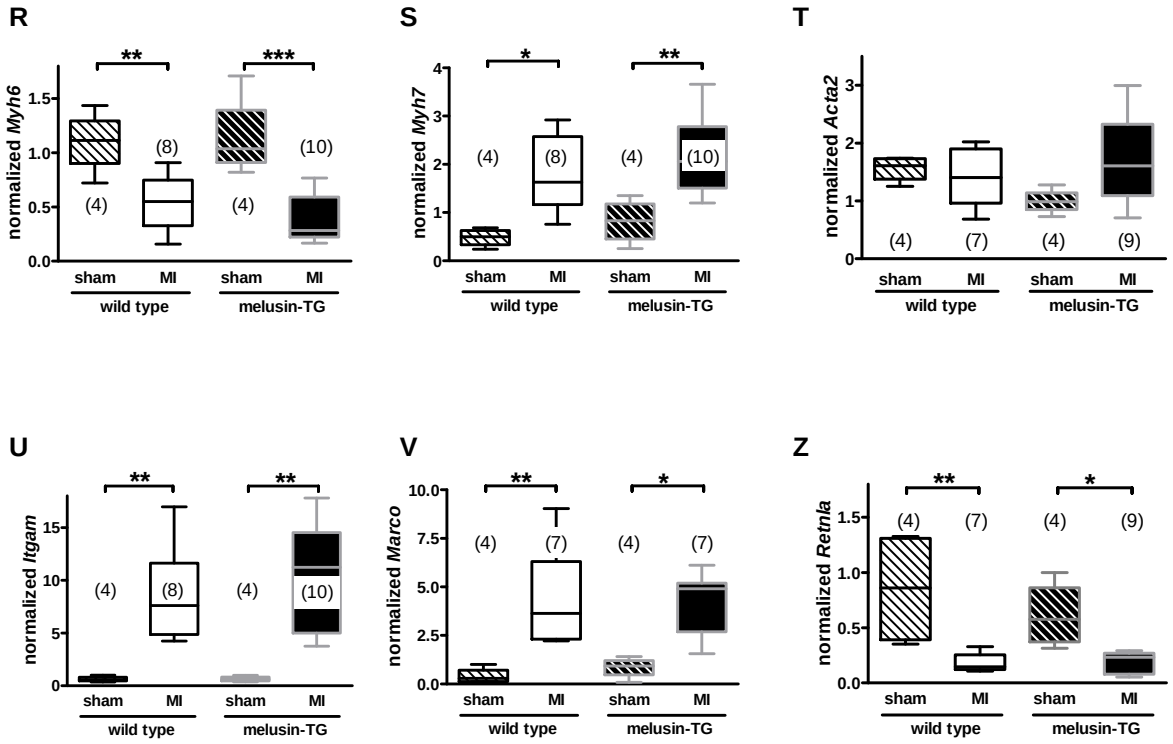
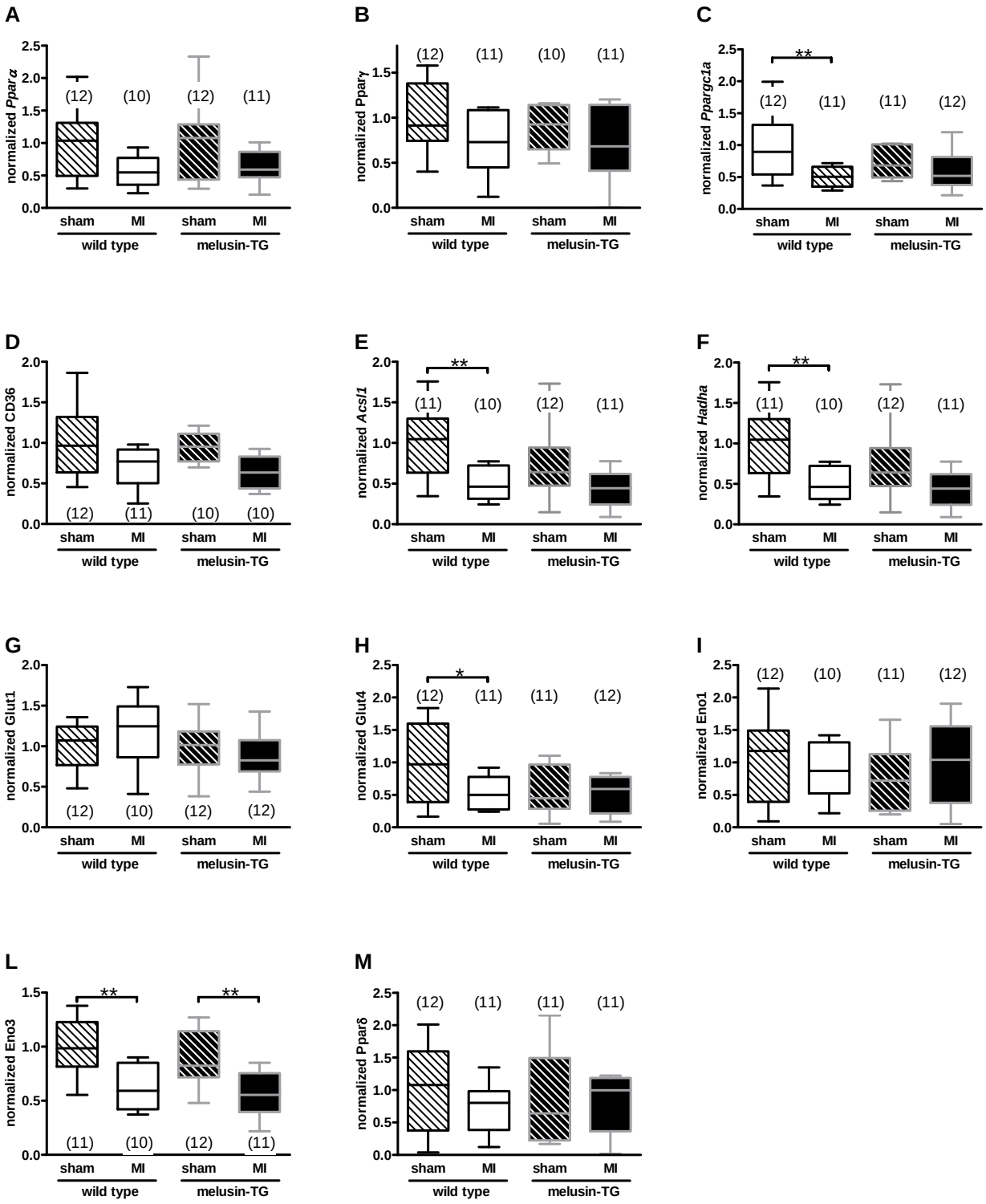
Q
normalized Tnf
sham
MI
wild type
melusin-TG

Figure S1



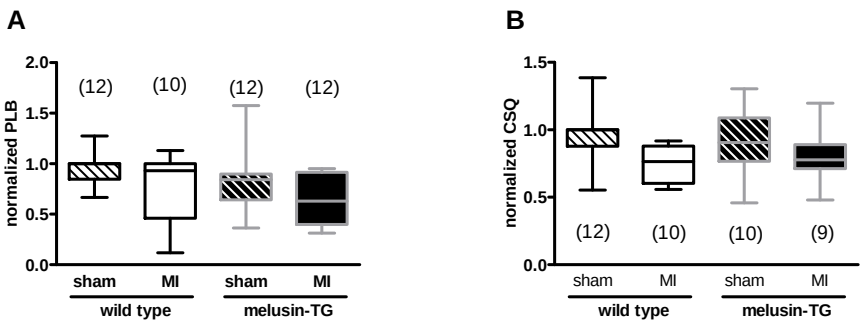
Supplementary Figure 1
mRNA expression of hypertrophic markers, cytokines and growth factors 3 days after MI.

Figure S2



Supplementary Figure 2
mRNA expression of metabolic enzymes 3 days after MI.

Figure S3



Supplementary Figure 3
Western blot analysis of phosphorylated phospholamban and calsequestrin 12 days after MI.