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Supplementary Information for

Resolving titin's lifecycle and the spatial organization of protein turnover in mouse cardiomyocytes

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Supplementary Material and Methods

Generation of the DsRed(Z)-knock-in mouse and double heterozygous titin-DsRed/titin-eGFP(M) mice. A targeting vector to insert the DsRed cDNA into titin´s exon 28 (Z-disk) was generated using standard procedures. The neomycin (Neo) resistance cassette was integrated into the 3' untranslated region (Fig. 1). The targeting vector was assembled and verified by restriction digest and sequence analysis. ES cells were transfected by electroporation with the linearized targeting vector and selected with G418 as described previously (1). Individual colonies were expanded and analyzed for homologous recombination by PCR (0.6% of neomycin resistant clones). After blastocyst injection and germline transmission, the Neo cassette was excised using the Flp FRT recombination system. Animals were backcrossed on a 129/S6 background and for each experiment littermates were used (age and sex matched). To generate 100% double-heterozygous titin-DsRed(Z)/titin-eGFP(M), we mated homozygous mice of each strain. Ttitin-eGFP(M) has been described previously (2). Double heterozygous offspring express GFP labeled titin from one allele and DsRed labelled titin from the other allele. Both, full length titins (labeled with either fluorophore) and truncated isoforms or proteolytic fragments (that retain the Z-disk or M-band region with DsRed or eGFP, respectively) contribute to the fluorescence signal.

Genotyping. Genomic DNA was prepared from ear-tags following standard procedures. Proper integration of the fluorophores was verified by PCR using specific primers. The DsRed construct into the titin locus was monitored by PCR using primers 3 neoflox (5'-TCGACTAGAGGATCAGCTTGGGCTG -3') and DsRed Geno (5'-ATAGAGACCTTGCTTTGCCTGTG -3'). Excision of the Neo selection in the DsRed animals was confirmed using primers fDsRedrecF (5'-

CAGCATCATGGTAAAGGCCATCAA -3') and rDsRedrecF (5'-CATTCAAATGTTGCCATGGTGTCC-3'). The Flp recombinase allele was detected using primers Flp-for (5'-GTCACTGCAGTTTAAATACAAGACG-3') and Flp-rev (5'-GTTGCGCTAAAGAAGTATATGTGCC-3'). Titin wild-type and titin eGFP loci were typed using primers Mex6SAi-for (5'-AGAACAACAAGGAAGATTCCACA-3'), SAK-rev (5'-TCTCAACCCACTGAGGCATA-3'), and eGFP-rev (5'-AGATGAACTTCAGGGTCAGCTTG-3').

Animal procedures. All experiments involving animals were carried out following the Guide for the Care and Use of Laboratory Animals of the German animal welfare act. Mice were age and sex matched and were sacrificed by cervical dislocation. The body weight of wild-type, heterozygous, and homozygous titin-DsRed or double heterozygous titin-DsRed/eGFP mice was determined. Hearts were collected and the weight was measured to calculate the heart weight to body weight ratio.

SDS-agarose electrophoresis and Western Blot. Protein lysates were prepared and analysed as described previously (3). In brief, lysates were homogenized in titin sample buffer (8 M urea, 2 M thiourea, 3% SDS, 0.05 M Tris-HCl, 0.03% bromophenol blue, 75 mM DTT, pH 6.8), extracted for 30 minutes and debris was removed by centrifugation. Proteins were separated on agarose gels and blotted on PVDF membranes. Primary antibodies were purchased from Invitrogen (anti-GFP; A11120) and from Clonetech (anti-dsRed monomer; 632496). The secondary HRP conjugated antibody was detected by chemiluminescence staining with ECL (Supersignal West Femto Chemiluminescent Substrate; Pierce Chemical Co.) or by fluorescence staining with IRDye 680LT and IRDye 800CW (Odyssey, Li-COOR infared detection system). Each experiment was reproduced at least twice independently.

Real-time PCR. Heart tissue of all genotypes was collected, snap frozen and grinded. Total RNA was isolated from tissue powder with Trizol followed by a cleaning step with the Qiagen RNeasy isolation Kit. To convert RNA into complementary DNA (cDNA), a reversed transcription PCR with a viral RNA-dependent DNA polymerase was performed using the RNA to cDNA Kit from Applied Biosystem or ThermoScientificTM RevertAid RT Kit according to manufacturer's specifications.

Immunofluorescence staining. Hearts from DsRed homozygous and DsRed/eGFP double heterozygous animals were dissected and prepared for immunofluorescence staining as described previously (2). Cryosections were permeabilized and blocked with 10% goat serum, 0.3% Triton X-100 and 0.2% BSA in PBS for 2h. Embryonic and adult cardiomyocytes were fixed with 4% PFA at room temperature for 10 min followed by permabilization and blocking as above.

The incubation with the primary antibody (1:100 anti-α-actinin, Sigma-Aldrich; 1:200 anti-titin M8M9, Siegfried Labeit; 1:50 anti-RPLP0; Sigma-Aldrich; 1:50 anti-20s proteasome, ThermoFisher; 1:50 anti-MTCO1, Abcam; 1:250 anit-TDP-43, Abcam) was performed at 4°C overnight followed by labelling with a fluorescent secondary antibody (1:500 to 1:1000 goat anti-mouse or goat anti-rabbit IgGAlexa Fluor 647 from Invitrogen) for 2h at room temperature. Cells stained for STED imaging were labelled with photostable fluorescent secondary antibodies from Abberior (STAR 635P goat anti-rabbit and STAR 580 goat anti-mouse, 1:500 in PBS).

Images were acquired with a confocal laser-scanning microscope (LSM710, Carl Zeiss) with a Plan-Apochromat 63x/1.4oil Ph3 objective. Super-resolution imaging was performed with a 3D-STED microscope (Abberior Instruments) and a 100x oil objective (UPLANSAPO) or a TCS SP8 STED microscope (Leica) with a HC PL APO C2S 100x oil objective (1.4 NA) as described previously (4). For imaging with the Leica STED system,

Abberior STAR 635P was excited at a wavelength of 635 nm and fluorescence was detected between 650 nm and 700 nm. Abberior STAR 580 was excited at 580 nm and fluorescence was detected between 590 nm and 630 nm. For the Abberior system the fluorescence excited at 635 nm was detected between 605 and 625 nm and the fluorescence excited at 580 nm between 650 and 720 nm. For both dyes and systems, STED imaging was performed with a 775 nm depletion laser. Imaging with the Leica STED system was executed with a gating between 0.5 ns and 6 ns, and images were acquired with a pixel size of 23 nm x 23 nm and a scanning speed of 600 Hz (pixel dwell time 0.4 µs). With the Abberior system the depletion laser was used with a laser power of 15% and images were made with a pixel size of 15 nm x 15 nm.

Ribosomes and proteasomes are quantified with Fiji software using the “analyse particle” option. A binary image was created from the raw image file by adjusting the threshold in a way that only signal dots are marked but not the background. For the same stainings we applied identical thresholds. To account for overlying particles, we applied the watershed option and to reduce background we did not consider particles smaller than 3 pixels. The number of particles was normalized to the area.

Single molecule fluorescence *in situ* hybridization (smFISH). Adult cardiomyocytes were fixed with 2% PFA for 10 min at room temperature followed by permeabilization with 70% ethanol overnight at 4°C. After equilibration in washing buffer (10% formamide and 2x SSC) for 15 min at 37°C the hybridization is performed for 16 h at 37°C with 100 nM of the probe diluted in hybridization buffer (10% formamide and 8% dextrane sulfate). Custom probe sets for single molecule FISH labeled with CalFluor-610 and Quasar-570 were designed using *Stellaris RNA FISH probe designer* (Biosearch Technologies). Washing for 30 min at 37°C was followed by DAPI staining (1:2000 in washing buffer) for 10 min at 37°C. All solutions were prepared with nuclease-free water.

For imaging on the next day we used a widefield microscope (Nikon Eclipse Ti) and a PlanApoλ 100x oil Ph3 objective. The samples were illuminated with the Prior Lumen 200 system and imaged with the filters forDAPI (Ex: 387/11, Em: 447/60, beam splitter: HC BS 409), GFP (Ex: 470/40, Em: 525/50, BS: T 495 LPXR), Quasar-570 (Ex: 534/20, Em: 572/28, BS: HC BS 552) and CalFluor-610 (Ex: 580/25, Em: 625/30, BS: T 600 LPXR)and the Andor DU888 camera. For each image 21 z-stacks with 0.3 µm steps are taken and later on processed with Fiji software (5). Background was reduced by subtraction with a Median filtered (50 px) copy of the image for all channels except of the DAPI channel. Z stacks were projected with maximal intensity. To visualize the relative localization of the RNA spots in the sarcomere four regions (50 x 60-90 px) of each cell were selected and straightened. These regions were stitched pairwise with the maximal intensity fusion method to increase the number of spots per region for plotting (6). Background spots were identified as fluorescence signals that extend across channels with complete colocalization.

Isolation of ES-cells and differentiation into cardiomyocytes. Embryonic stem cells were isolated from blastocysts at E3.5 of pregnancy. The uterus was flushed with PBS and the blastocysts were cultured on inactivated fibroblasts (Myotmycin C) with one blastocyst per well in ES cell medium (NDiffN2B27 with 1% penicillin-streptomycin, 3 mM PD and 5 mM CHIR - both axon MedChem). After outgrowth of the inner cell mass, cells were picked and dissociated with accutase and cultivated on inactivated fibroblast until ES cell colonies appeared. ES cells were then cultivated in ES cell medium (DMEM with 15% FBS, 1% penicillin-streptomycin, 1% non-essential amino acids, 1% β-mercaptoethanol and LIF). For differentiation into cardiomyocytes the ES cells were dissociated with trypsin and fibroblasts were removed by pre-plating for 2h. The medium contained no LIF and 15%

FBS for differentiation. ES cells were transferred in 20 µl drops of medium with 400 – 800 cells per drop on the lid of a PBS filled dish and incubated upside down (hanging drops). After 2 – 3 days embryoid bodies had formed and were maintained in suspension until they were transferred on galantine-coated coverslips or ibidi dishes for analysis.

Isolation of embryonic and adult cardiomyocytes. Embryonic cardiomyocytes were isolated at E13.5 as described previously (2) and seeded on gelatin-coated coverslips for confocal and super-resolution imaging and on coated ibidi dishes for *live* imaging.

Adult cardiomyocytes were isolated from hearts of 8 – 12 weeks old mice. The aorta was cannulated and perfused with perfusion buffer (120.4 mM NaCl, 14.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO , 1.2 mM $MgSO_4$, 10 mM Na-HEPES, 4.6 mM $NaHCO_3$, 30 mM Taurin, 10 mM BDM, 5.5 mM glucose) at 37°C with 4 ml/min. After 1 min perfusion solution was switched to perfusion buffer with Collagenase Type II (500 U/ml and 40 µM $CaCl_2$). The ventricles were collected and dissected before the digestion was stopped with stopping buffer (10% FBS in perfusion buffer with 12.5 µM $CaCl_2$). Cells were separated by resuspension and washed with stopping buffer after sedimentation. They were seeded in perfusion buffer and fixed after 1h.

Cell length and sarcomere length measurements. For evaluation of *in vitro* contractile properties, we assessed cell and sarcomere shortening in intact cardiomyocytes isolated from. Cells were isolated from WT and RFP-homozygous hearts at 8 – 9 weeks as described above with the following modifications. We used Liberase/ Trypsin (0.25/ 0.14 mg/ml) for digestion and calcium was reintroduced stepwise to a final concentration of 1 mM. Cells were placed in a chamber mounted on the stage of an inverted microscope (Leica DMI3000 B) in extracellular solution containing (in mM): 130 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 15 glucose, 10 HEPES, at pH 7.4 at room temperature. The myocytes being studied were scanned with a camera at 250 Hz under a 40x objective. Field stimulation

(10 V) was performed using a MyoPacer (IonOptix, Milton, USA) to contract at a frequency of 1 Hz. Changes in cell length during shortening and re-lengthening was captured using a video-based edge-detection system (IonOptix, Milton, MA) and converted to digital signal before being analyzed with IonWizard (Version 6.6, IonOptix, Milton, USA) software. Sarcomere length was assessed by calculating and inverting the fundamental frequency of the Fourier spectrum into sarcomere length. Contracting cardiomyocytes were selected for recording according to the following criteria: 1.) rectangular shape with sharp edges and 2.) cell length > 80 µm. For analysis cardiomyocytes with 8 -100 noise-less contractions were accepted.

Myoseverin treatment. Embryonic cardiomyocytes were isolated as described (2) and live imaging was performed at the DeltaVision Elite microscope (GE healthcare). During imaging, cells were kept under 37°C and 5% CO_2. Myoseverin was diluted in DMSO. The imaging process started immediately after changing the medium to FluoroBrite (ThermoFisher) with 20 µM Myoseverin.

Proteasome and protease inhibitor treatment. Adult cardiomyocytes were isolated as described above and seeded in myocyte plating medium (MEM, 5% FBS, 1% penicillin-streptomycin, 2 mM L-glutamine, 25 µM blebbistatin). After attachment for 1 h medium was changed to medium containing 1 µM of the proteasome inhibitor MG132 and/or 20 µM of the protease inhibitor E64d. Since both were diluted in DMSO, an additional control with DMSO was performed. The cells were incubated for 6 h under 37°C and 5% CO_2 before they were fixed.

Fluorescence recovery after photobleaching. FRAP experiments were performed as previously described (2) with the following modifications. Embryonic cardiomyocytes were isolated from homozygous titin-eGFP(M) and homozygous titin-dsRed(Z) animals. The experiments were performed on the DeltaVision Elite microscope with the 60x oil objective

(NA 1.42). EGFP fluorescence was photobleached with a 488 nm laser at 25% intensity for 0.1 s and dsRed fluorescence with a 561 nm laser at 100% intensity for 0.25 s. The region of interest (ROI) covered 2 sarcomeres and the fluorescence recovery was followed over 14 h with imaging every 30 min for the first 2h and then every hour. For each genotype 5 experiments were performed (n = 5) with 3 cells per experiment. Fluorescence intensity was measured at the respective integration sites at up to 5 regions per cell. Normalized recovery curves were calculated as described previously and fitted with a one phase association curve using GraphPad Prism. From this curve the exchange half-life was obtained as the time point where 50% of the maximal signal had returned. For cells with a biphasic recovery, we fitted a two phase association curve:

$$y(t) = (y_0 + Span_{fast} * \left(1 - e^{-K_{fast} * x}\right) + Span_{slow} * (1 - e^{-K_{slow} * x})$$

Additionally the mobile fraction was calculated (2). FRAP data are represented as mean of the 5 individual experiments or as values obtained from a single cell.

Statistical analysis. For statistical analysis we used GraphPad Prism 5.0. Results are expressed as mean ± SEM and significance is indicated as $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). Differences between two groups were evaluated using t-test (unpaired, two-tailed) and between three and more groups with One-way-ANOVA. Two-way ANOVA was used, if groups were affected by two different factors. After ANOVA tests we applied the Bonferroni post test. Additional information for each experiment is provided in the respective figure legend.

Supplementary References

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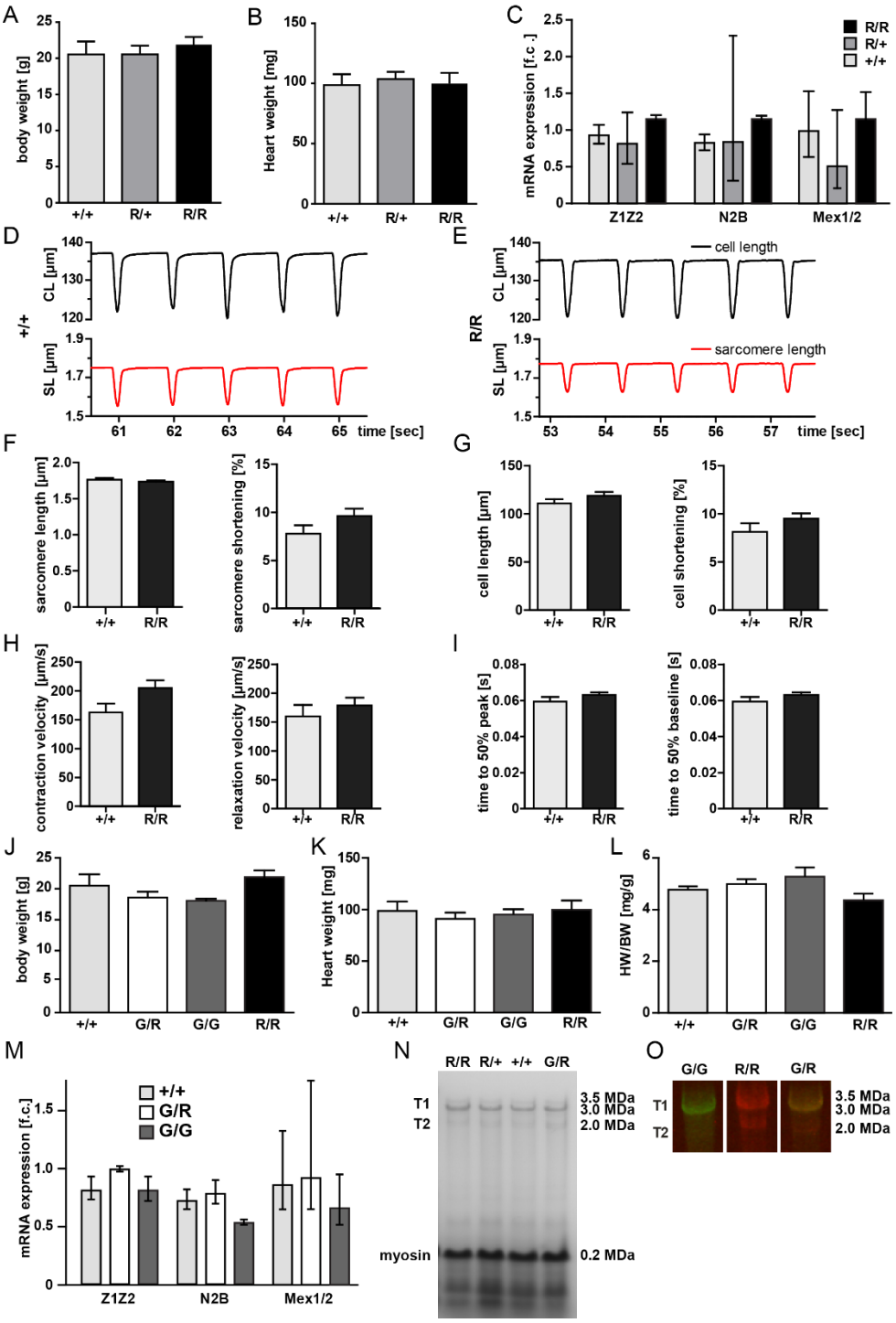
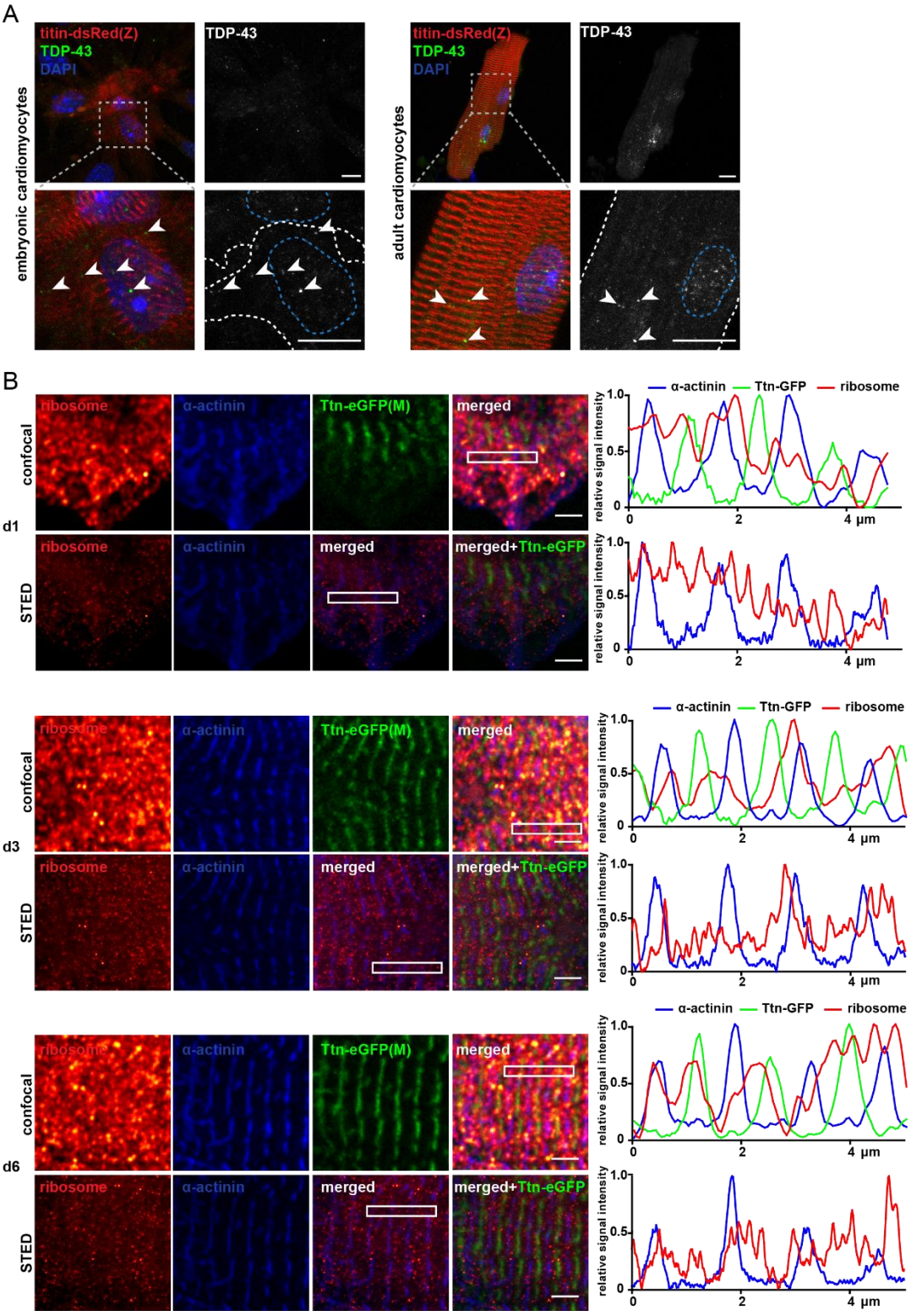
Figures

Supplement Figure 1, related to Figure 1



Supplementary Figure 1: titin-DsRed(Z)/titin-eGFP(M) double heterozygous animals are phenotypically normal. (*A, B*) Heart and body weight are unchanged in heterozygous (R/+) or homozygous (R/R) DsRed knockin animals. (*C*) The introduction of the DsRed sequence does not alter titin isoform expression with similar levels of Z-disk, N2B, and M-band exons. (*D, E*) Representative tracings of cell and sarcomere shortening induced by pulses at 1 Hz (10 V) in 2.0 mM Ca^{2+} from wild type and RFP-positive cardiomyocytes. (*F-I*) dsRed homozygous cardiomyocytes do not differ in diastolic sarcomere and cell length and shortening (*F, G*)**,** contraction and relaxation velocity (*H*) or time to 50% of peak and time to 50% baseline (*L*). There was no significant difference between DsRed homozygous cardiomyocytes (3 mice, n = 35 cells) and WT (+/+) cardiomyocytes (3 mice, n = 18 cells). (*J*, *K*, *L*) Double heterozygous animals (G/R) have normal body weight, heart weight, and heart body-weight ratios as compared to wildtype (+/+) and homozygous animals (G/G or R/R). +, wildtype allele; R, titin-DsRed(Z) allele; G, titin-eGFP(M) allele. (*M*) The eGFP allele in double heterozygous or homozygous animals does not alter titin isoform expression. (*N*) On the protein level, cardiac titin expression and isoform composition is unchanged in animals carrying the titin-DsRed(Z) allele. T1 full-length titin isoforms, T2 proteolytic fragment that contains the titin Z-disk but not M-band region. (*O*) Western blot to confirm the presence of DsRed and eGFP domains with only DsRed present in the N-terminal T2 fragment.

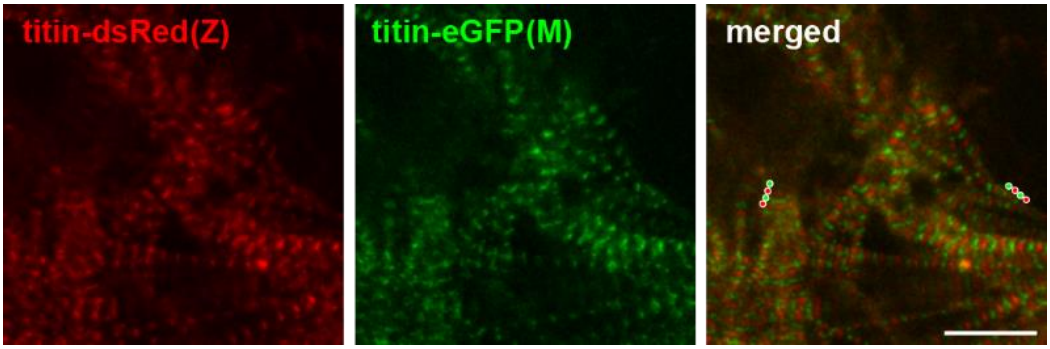
Supplement Figure 2



Supplementary Figure 2: Localization of ribosomes in embryonic cardiomyocytes.

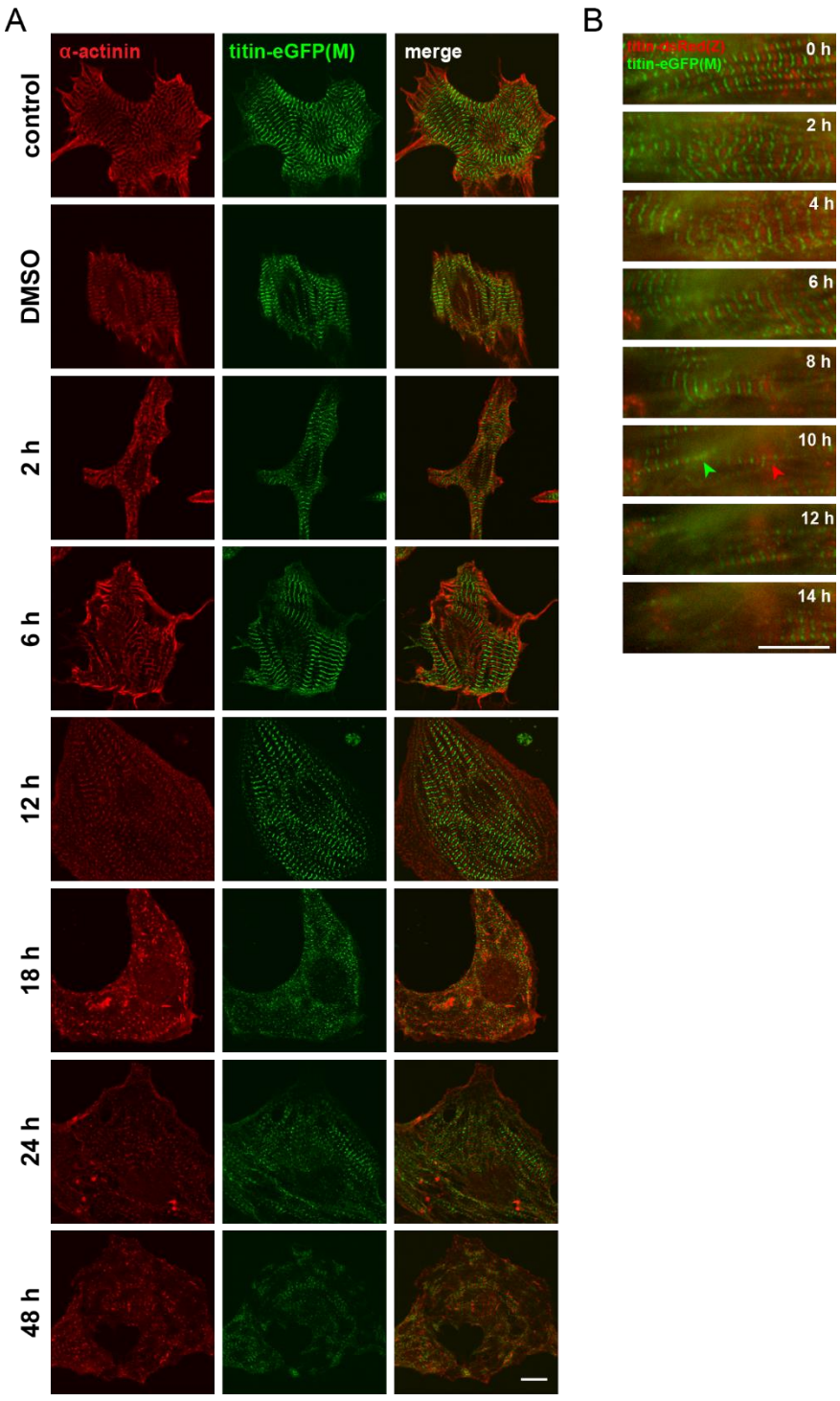
(*A*) The RNA binding protein TDP-43 is expressed in cardiomyocytes and more abundant in the nucleus as comared to the cytoplasm. The few sarcomeric TDP-43 spots in adult cardiomyocytes localize towards the Z-disk. (*B*) As embryonic cardiomyocytes mature from day 1 through day 6 in culture (d1 through d6), the ribosome signal remains diffuse. Traces are derived from the boxed areas. There is no periodicity in the ribosome traces that aligns to the periodicity of titin and alpha-actinin. Scale bar 10 µm (overview); scale 2 µm (magnification).

Supplement Figure 3



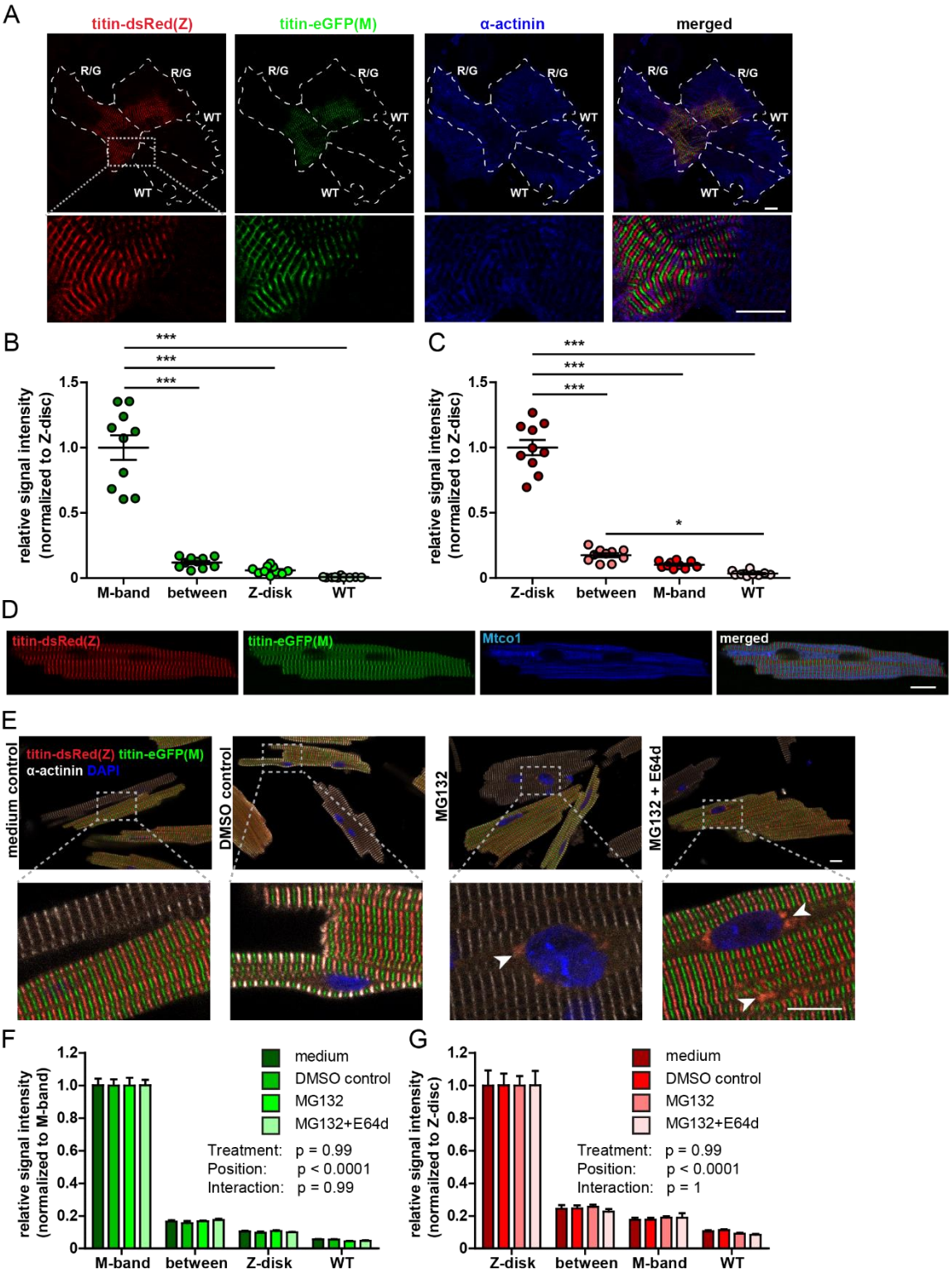
Supplementary Figure 3: Integration of titin in cardiomyocytes differentiated from ES cells. Myofilament assembly in ES-derived cardiomyocytes, which integrate titin-DsRed(Z) and titin-eGFP(M) in parallel as newly formed filaments (emerging sarcomeres labelled with alternating red and green dots). Scale bar 10 µm.

Supplement Figure 4



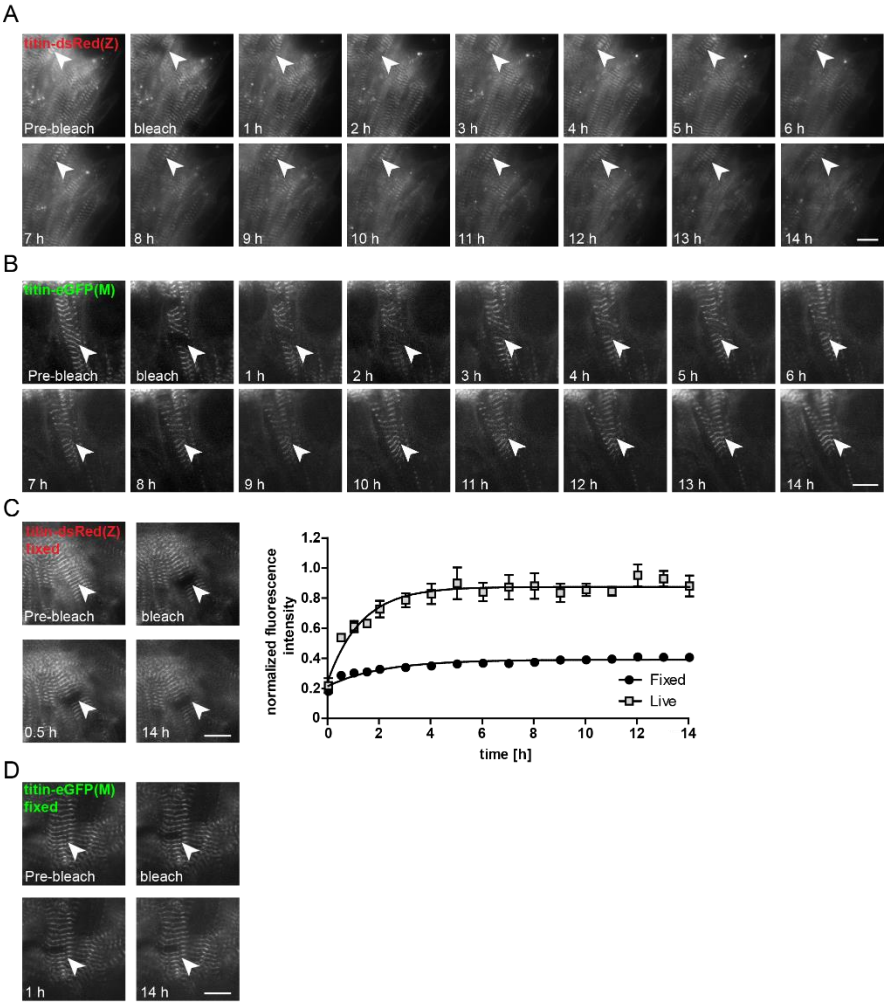
Supplementary Figure 4: Disassembly of mature sarcomeres after myoseverin treatment. (*A*) Embryonic titin-eGFP(M) cardiomyocytes were treated with myoseverin, fixed, and stained for α-actinin. Disassembly was present from 18h after treatment. (*B*) Live imaging of sarcomere disassembly in double heterozygous embryonic cardiomyocytes. Both titin-DsRed(Z) and titin-eGFP(M) doublets exist (red and green arrows).

Supplement Figure 5



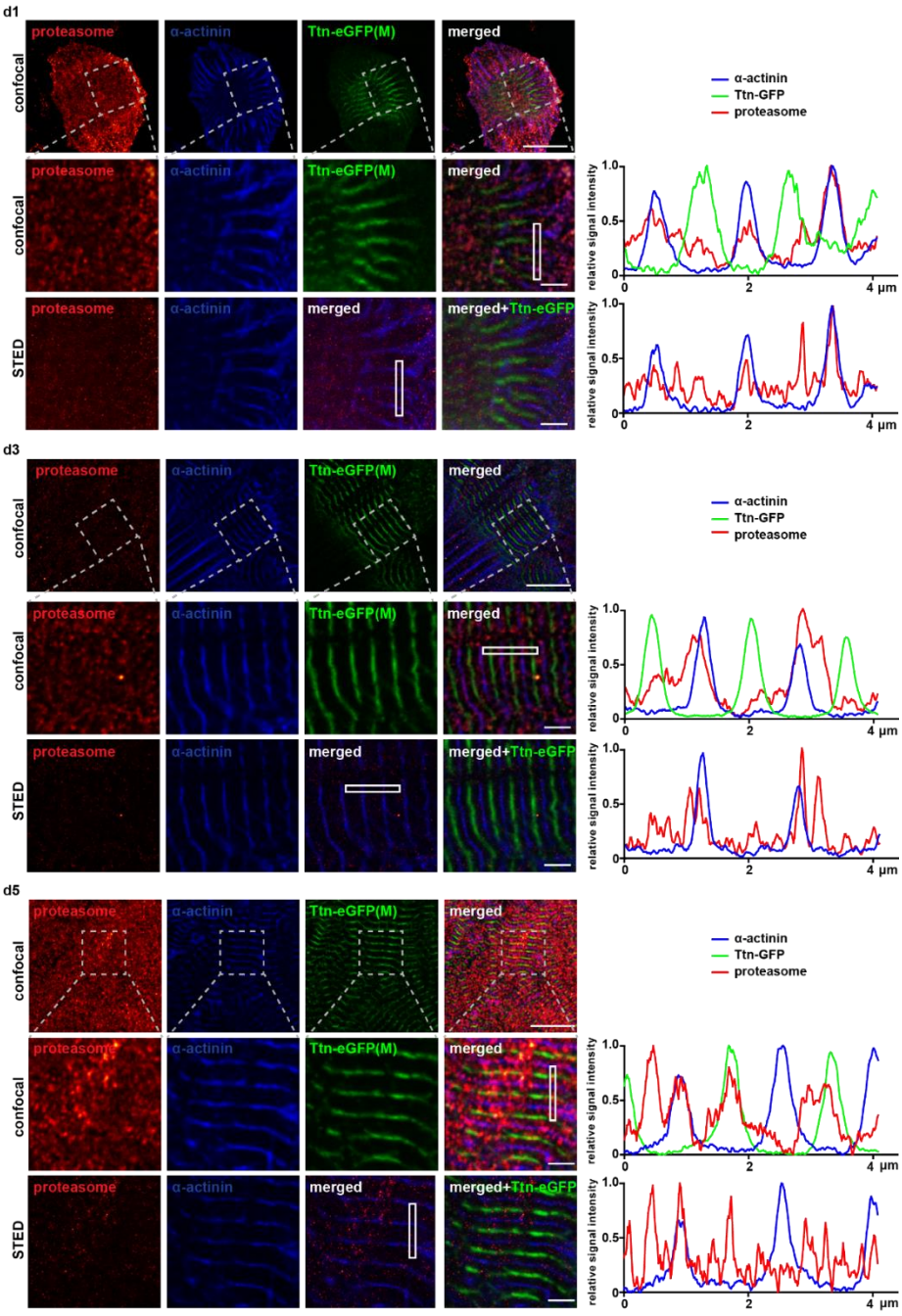
Supplementary Figure 5: **Background autofluorescence is elevated in areas with high mitochondrial density.** (*A*) Co-cultivation of double heterozygous DsRed/eGFP and wildtype embryonic cardiomyocytes to compare background fluorescence in the same image. (*B*, *C*) Normalized fluorescent signal between integration sites (outside the DsRed and eGFP signal at Z-disk and M-band, respectively) is above background as compared to autofluorescence of wildtype cells. One-Way-ANOVA with Bonferroni post-test, n = 10, *p<0.05; **p<0.01; ***p<0.01. (*D*) DsRed/eGFP adult cardiomyocyte with background signal along the long axis corresponds to the location of mitochondria. (*E*) In adult cardiomyocytes treated for 6 h with the proteasome inhibitor MG132 and the protease inhibitor E64d aggregates with red autofluorescence appear. (*F*, *G*) The treatment does not affect the fluorescence signal between the integration sites of both fluorophores. Two-way-ANOVA (n=10 per group). Scale bar 10 µm.

Supplement Figure 6



Supplementary Figure 6: FRAP-timeline of Z-disk and M-band titin. (*A*, *B*) Titin recovery after photobleachinig titin-DsRed(Z) and titin-EGFP(M) over 14 h. (*C*, *D*) In fixed cells there is no recovery within 14 h after photobleaching. Size bars 10 µm.

Supplement Figure 7



Supplementary Figure 7: Localization of proteasomes in embryonic cardiomyocytes. In embryonic cardiomyocytes matured from day 1 through 5 in culture,

the proteasome signal remains diffuse. There is no periodicity in the proteasome traces that aligns to the periodicity of titin and α-actinin. Scale bar 10 µm (overview); scale bar 2 µm (magnification).

Supplementary Tables

Table S1: Comparison of titin kinetics at Z-disk and M-band

Cell type	Mf [%]	$t_{1/2}$ [h]
Titin-dsRed(Z)	85.2 ± 6.8	0.88 ± 0.18
Titin-eGFP(M)	64.3 ± 6.2	3.65 ± 0.59

Mf: Mobile fraction; $t_{1/2}$: exchange half-life of titin. Titin-dsRed(Z): cells homozygous for dsRed labeled titin; Titin-eGFP(M): cells homozygous for eGFP labeled titin.

Table S2: Heterogeneity of titin dynamics between single cells

Cell type	Classification	Occurrence	$t_{1/2}$ [h]
Titin-dsRed(Z)	Fast	60%	0.61 ± 0.10
Titin-dsRed(Z)	Biphasic	20%	Fast phase: 0.13 ± 0.02 Slow phase: 4.42 ± 2.92
Titin-dsRed(Z)	Slow	20%	1.44 ± 0.21
Titin-eGFP(M)	Fast	80%	2.49 ± 0.20
Titin-eGFP(M)	Slow	20%	5.55 ± 0.32

$t_{1/2}$: exchange half-life of titin; Titin-dsRed(Z): cells homozygous for dsRed labeled titin; Titin-eGFP(M): cells homozygous for eGFP labeled titin.