

2016
MÁJUS 25-26.

STRAUB-NAPOK

MEGHÍVÓ



MTA SZEGEDI BIOLÓGIAI KUTATÓKÖZPONT
AZ EURÓPAI UNIÓ KIVÁLÓSÁGI KÖZPONTJA
SZEGED, TEMESVÁRI KRT. 62., NAGYELŐADÓ



Retromer is required for autophagy through maintaining proper lysosome function in *Drosophila*

Tamás Maruzs^{1,2}, Péter Lőrincz¹, Zsuzsanna Szatmári¹, Szilvia Széplaki¹, Zoltán Sándor¹, Zolt Lakatos¹, Gina Puska¹, Gábor Juhász^{1,2} and Miklós Sass¹

(¹Department of Anatomy, Cell and Developmental Biology,

Eötvös Loránd University, Budapest;

²Institute of Genetics, BRC HAS, Szeged)

Stem cell derived *rybp null* mutant cardiomyocytes lack functional sarcomeres

Viktória Szabó and Melinda K. Pírity

(Institute of Genetics, BRC HAS, Szeged)

Alteration of retinoic acid signaling in *rybp null* mutant neural cultures

Enikő Sutus, Gergő Kovács and Melinda K. Pírity

(Institute of Genetics, BRC HAS, Szeged)

A doxycycline-regulable gene expression system in B16-F10 mouse melanoma cell line

Dávid Pusztai¹, Péter Germán¹, Katalin Hegedűs¹, Ildikó Fekete¹,

Ibolya Horváth², László Vígh² and Lajos Mátés¹

(¹Institute of Genetics, BRC HAS, Szeged;

²Institute of Biochemistry, BRC HAS, Szeged)

Somatic-transgenic mouse model to introduce random genome-wide deletions

Gergely Imre¹, Liza Hudoba¹, Andrea Nagy¹, Bálint Balázs³, István Nagy^{2,3}, Thomas Rülcke⁴ and Lajos Mátés¹

(¹Institute of Genetics, BRC HAS, Szeged;

²Sequencing Laboratory, BRC HAS, Szeged;

³SeqOmics Biotechnology Ltd., Mórahalom;

⁴Institute of Laboratory Animal Science, University of Veterinary Medicine, Vienna, Austria)

Neuronal cytoskeleton regulation during axonal growth in *Drosophila*

Tamás Mészáros¹, István Földi², Szilárd Szikora², Rita Gombos²,

Ede Migh² and József Mihály²

(¹Universitatea Babeş-Bolyai, Cluj-Napoca, Romania;

²Institute of Genetics, BRC HAS, Szeged)

INSTITUTE OF PLANT BIOLOGY

Arabidopsis retinoblastoma-related (RBR) acts on chloroplast biogenesis and differentiation in an E2FC-dependent manner

Eszter Molnár¹, Beatrix Horváth^{2,4}, Ottó Zsíros¹, Gábor V. Horváth¹, Árpád Párducz³, Ben Scheres², Győző Garab¹, László Bögre⁴, and Zoltán Magyar¹

(¹Institute of Plant Biology, BRC HAS, Szeged;

²Wageningen University, Wageningen, The Netherlands;

³Institute of Biophysics, BRC HAS, Szeged;

⁴Royal Holloway, School of Biological Sciences, University of London, Egham, Surrey, UK)

The Arabidopsis cell cycle regulator E2FB controls seed and early seedling development through the regulation of LEC2 and WRI1 genes

Tünde Levicky¹, Áron Juhász¹, Anita Kovács¹, Mihály Kis¹, Zoltán Gombos¹, János Pauk² and Zoltán Magyar¹

(¹Institute of Plant Biology, BRC HAS, Szeged;

²Cereal Research Non-Profit Ltd., Szeged, Hungary)

The Arabidopsis retinoblastoma-related (RBR) functions in sucrose-dependent manner

Márta Deli¹, Csaba Koncz^{1,2} and Zoltán Magyar¹

(¹Institute of Plant Biology, BRC HAS, Szeged;

²Max-Planck Institute for Plant Breeding Research, Cologne, Germany)

Regulation of ascorbate biosynthesis in the green alga *Chlamydomonas reinhardtii*

André Vidal Meireles¹, Juliane Neupert², Valéria Nagy¹, László Kovács¹, Laura Zsigmond¹, Laise Rosado de Souza², Alisdair R. Fernie², Ralph Bock² and Szilvia Z. Tóth¹

(¹Institute of Plant Biology, BRC HAS, Szeged;

²Max Planck Institute of Molecular Plant, Potsdam-Golm, Germany)

Stem cell derived *rybp null* mutant cardiomyocytes lack functional sarcomeres

Viktória Szabó, Melinda K. Pirity
Institute of Genetics, BRC-HAS, Szeged

ABSTRACT

Congenital heart defects arise at the early stage of embryonic development. They are caused by mutations and irregular changes of molecular pathways that govern embryonic heart development. Sarcomeric disfunction plays a central role in reduced cardiac pump function in heart failures. Our laboratory focuses on the Ring1- and Yy1-binding protein (Rybp; also known as DEDAF (Death effector domain associated factor), UniGene Mm.321633; MGI:1929059), which is a transcriptional regulator with essential role in early embryonic development [1]. We have previously showed that lack of Rybp in mouse embryonic stem (ES) cells impairs cardiac differentiation resulting cardiomyocytes (CMCs) without contractile activity [2]. The objective of this work was to characterise the *rybp null* mutant CMCs in respect to their sarcomere remodeling abilities in an *in vitro* ES cell based model system [3].

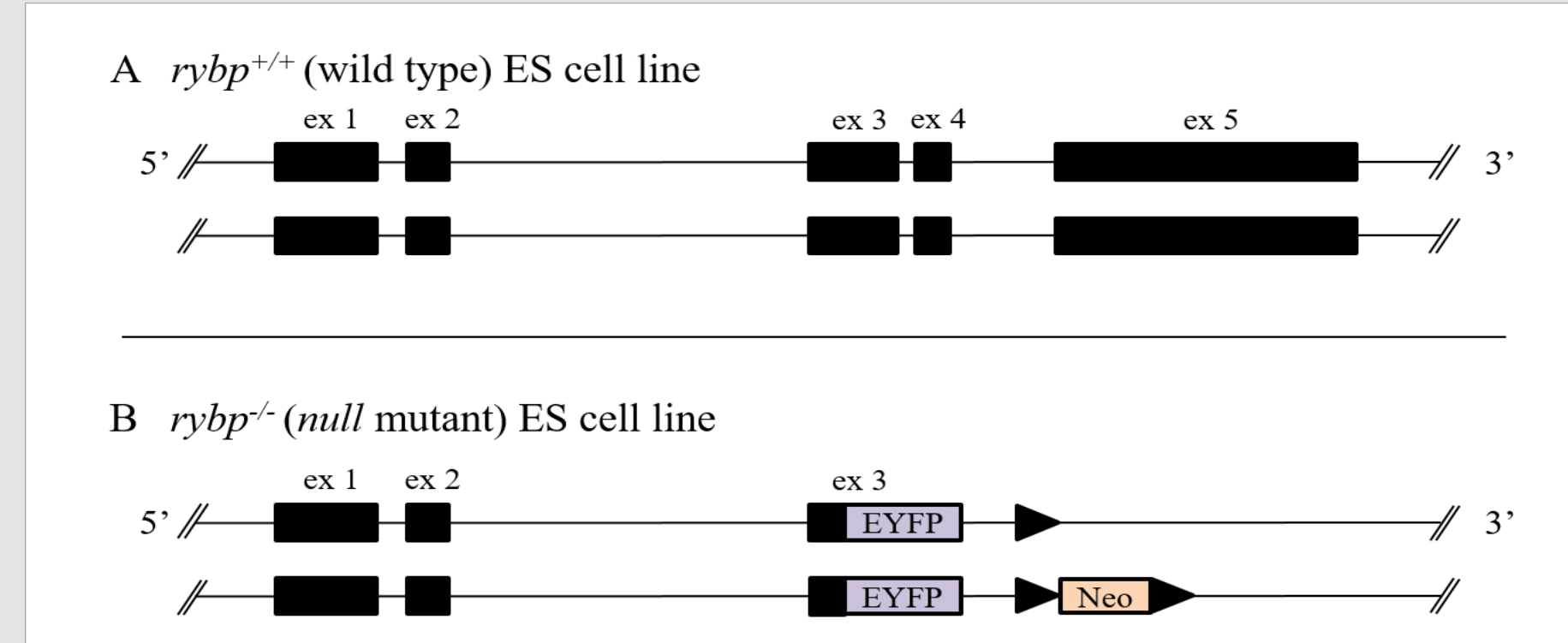


Figure 1: Stem Cell Lines (ex: exon; EYFP: Enhanced Yellow Fluorescent Protein; Neo: neomycin resistance gene; ►: loxP site)

METHODS

In vitro cardiac differentiation via hanging drop method:

For *in vitro* cardiac differentiation, embryoid bodies (EBs) were generated by the hanging drop (HD) method (2) [5]. The EBs were formed from *rybp*^{+/+} (wild type) (1A) [4] and *rybp*^{-/-} (null mutant) (1B) [1] mouse ES cells, using 1000 cells per drop (4x10⁴ cells/ml). Individual EBs were placed into 35 mm dishes containing 0.1 % gelatin coated cover-slips on the second day of differentiation (day2; d2). The differentiation medium was changed every second day. CMCs were grown for 21 days and observed for beating daily, under a phase-contrast microscope. Sampling time points were at day 0, 2, 7 and 14 for further gene expression studies (see below) and at day 14 and 21 for immunocytochemistry (see below) (2).

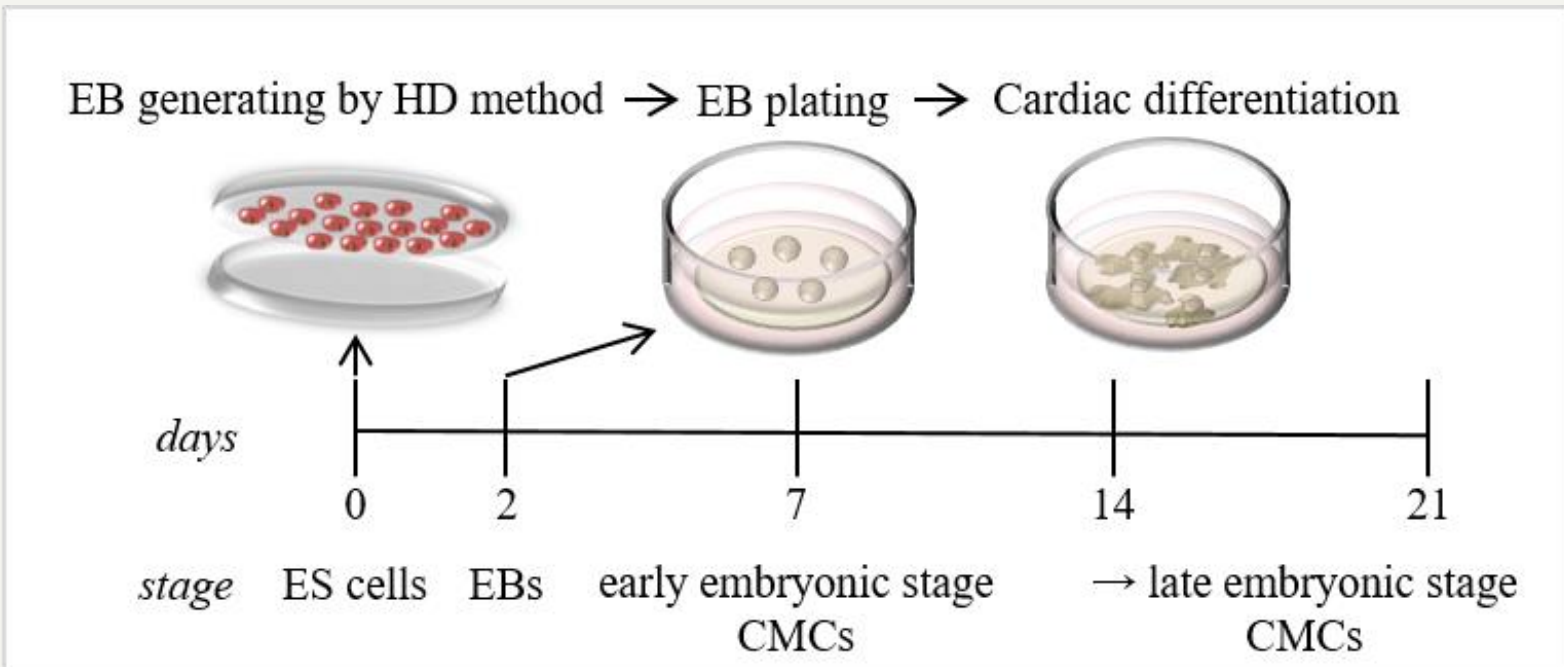


Figure 2: Time line of the cardiac differentiation

Quantitative Real-Time PCR (qRT-PCR):

RNA was isolated from the CMCs using Gene Jet RNA Purification Kit (Thermo Scientific, Cat. K0739) according to the manufacturer's protocol. RNA was translated to cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat. 4368814). Isolated cDNAs were used in qRT-PCR reactions to determine gene expression changes of sarcomere structure genes, which are thin filament associated (*tnni3*, *tnnt2*, *tpm1*, *tpm4*) and thick filament associated (*myh6*, *myh7*, *myom-1*, *ttn*). Reactions were performed in SYBR Green master mix (SYBR® Select Master Mix for CFX; Applied Biosystems, REF 4472942) using Bioer LineGeneK Real-time PCR System machine (Bioer, China). Relative gene expression changes were quantified using the $\Delta\Delta Ct$ method. The threshold (Ct) values for each gene were normalized to expression level of Hprt, as internal control. To calculate fold change, the values compared to undifferentiated samples (d0, *rybp*^{+/+}). Statistical method: T-test type 3. Values and means: $\pm SD$ p < 0.05 (*p < 0.05; **p < 0.01; ***p < 0.001). See results in Figure 4-9.

Immunocytochemistry (ICC) of CMCs:

CMCs were fixed with 4% (v/v) paraformaldehyde for 20 min at room temperature (RT), permeabilized with 0.2% Triton X-100 for 20 min at RT and blocked with blocking buffer (5% Bovine Serum Albumin) for 1h at RT, then sequentially incubated with the following primary antibodies at 4°C:

- Cardiac Troponin T (anti-cardiac Troponin T, Abcam, Cat. ab8295, 1:2000)
- Cardiac Troponin I (anti-cardiac Troponin I, DSHB, Cat. TI-1, 1:200)
- Tropomyosin (anti-Tropomyosin, BABRAHAM, Cat. BT-GB-141, 1:400)
- Sarcomeric Myosin (anti-Myosin-Sarcomere, DSHB, Cat. MF20, 1:200)
- Myomesin (anti-mMac Myomesin, DSHB, Cat. B4, 1:10)
- Titin (anti-Titin, DSHB, Cat. 9 D10, 1:200)

After this the cultures were incubated with fluorescent-labeled secondary antibodies for 1h:

- Alexa Fluor 568 D-A-M IgG (H+L) (Invitrogen, Cat. A-10037, 1:2000)
- Alexa Fluor 546 G-A-Rat IgG (H+L) (Invitrogen, Cat. A-11081, 1:600)

The cultures were rinsed in 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Cat. H-1200) for 20 min and mounted in 50% glycerol. The images were obtained by using Olympus Confocal LSM. Signals were quantified by Image J software analysis with RGB measure method. See results in Figure 4-9.

THE SARCOMERE

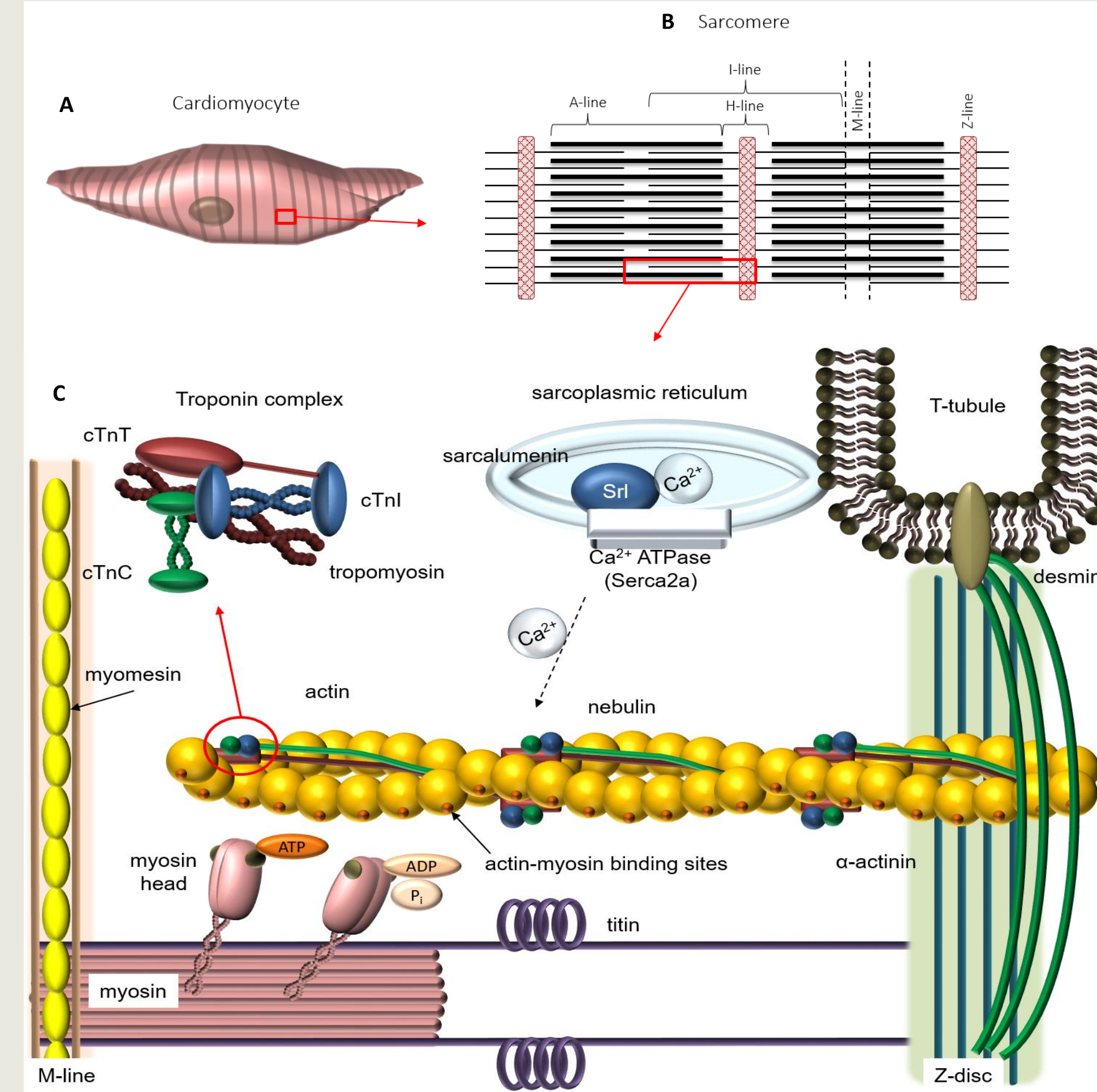


Figure 3: Sarcomere structure and contraction

Ca²⁺ ions bind to the troponin complex, tropomyosin moves away, then myosin can bind to actin-myosin binding sites on ATP-dependent manner. ATP hydrolyses, ADP and P_i get off, myosin heads bind to the actin, then a new ATP bind. While the Ca²⁺ ions are connected the myosin heads bind to the neighbouring actin globule (3C).

The CMCs are rod-shaped striated muscle cells with one or two nuclei. The red square represents a sarcomere unit (3A). The sarcomere is the smallest functional unit of the contractile muscle, which is important for generating mechanical force during contraction on a Ca²⁺-ATP-dependent way. The sarcomere is composed of thick (myosin)(thick line 3B; pink 3C) and thin filaments (actin)(thin line 3B; mustard yellow 3C) and associated proteins. The red square shows a half-sarcomere unit (3B).

Thick filament associated proteins (3C):

Myosin (pink) is a motor protein, it helps to relay energy and generating force [6]. **Myomesin** (yellow) is a myosin anchoring protein in the M-line and it connects with the titin [7]. **Titin** (lilac) is a molecular spring in the plastic elasticity of the striated muscle and helps for myosin to survive strain during contraction [8].

Thin filament associated proteins (3C):

The thin filaments are consisted of two **actin** (mustard yellow) strands, which contains actin-myosin binding sites (orange dots) [9]. **Nebulin** (vivid green) is a molecular template for the actin formation and specifies the actin filament length [10]. **Tropomyosin** (dark red) whips round the actin, it has essential role in the Ca²⁺-mediated contraction [11], because it contains the Ca²⁺ binding troponin complex, including cardiac **Troponin I** (blue), C (green) and T (red) [12]. **Alpha-actinin** (α -actinin) (aquamarine) anchors actin and titin are located in the Z-disc, crosslinks the neighbouring sarcomeres [13].

Other important sarcomere components (3C):

Extrasarcomeric **desmin** (green) stabilizes sarcomere on the Z-disc [14]. Sarcoplasmic reticulum (SR) (transparent blue) is essential for the contraction, as stores Ca²⁺ ions. SR has Ca²⁺ ATPase ion channels (Serca2a) (white). **Sarcalumenin** (blue) connects to the Serca2a and helps to transmit the Ca²⁺ ions through it [15].

RESULTS

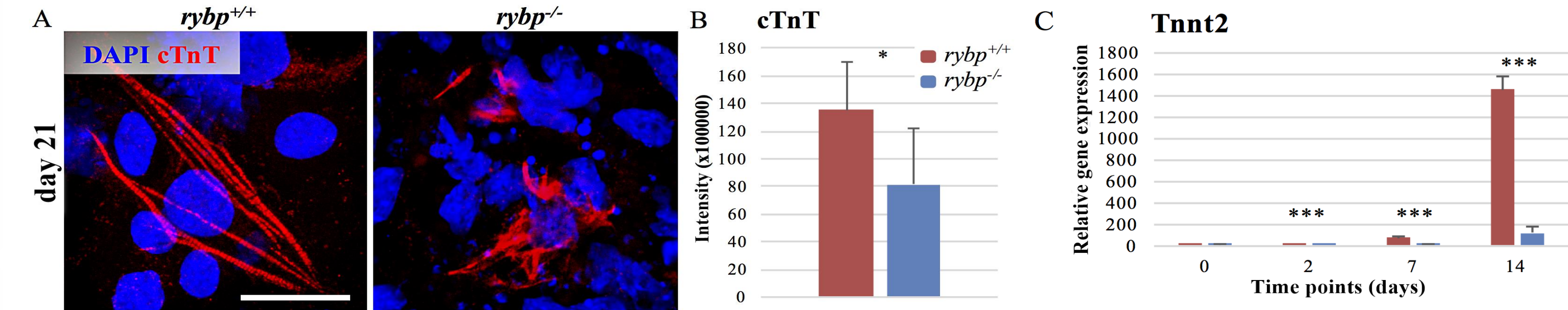


Figure 4: Cardiac Troponin T (cTnT) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. Anti-cardiac Troponin T antibody (red; 4A) staining showed that thin filaments were shorter and there were no sarcomeric structure in the *rybp*^{-/-} CMCs whilst it was clearly visible in the *rybp*^{+/+} cultures. Nuclei were stained with DAPI (blue; 4A). Intensity analysis revealed that the cTnT signal was half of the wild type level in the *rybp*^{-/-} mutants (4B). QRT-PCR analysis showed that the *tnnt2* gene was significantly downregulated in the *rybp*^{-/-} cells (4C). (Objective: 60x, zoom: 3.8x; Scale bar: 20 μ m) Deviation: $\pm SD$; Statistical method (t-test type 3): p<0,05 (*p<0,05; **p<0,01; ***p<0,001)

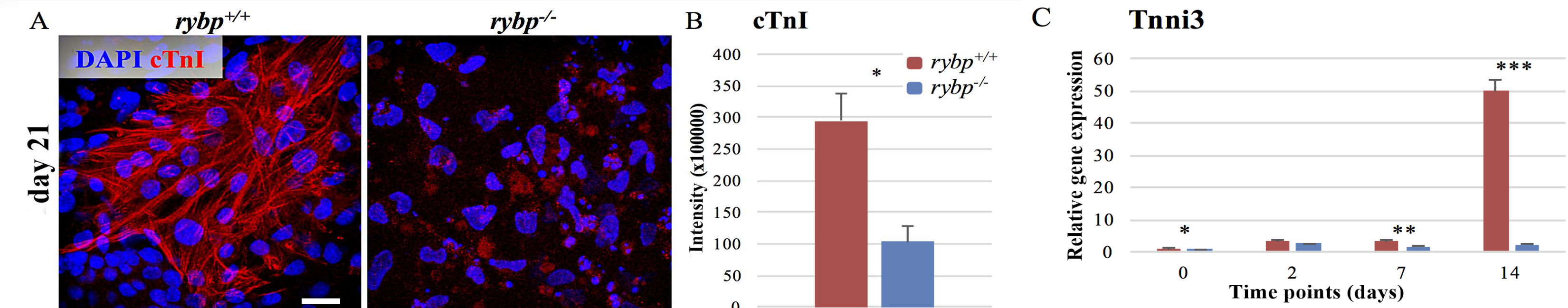


Figure 5: Cardiac Troponin I (cTnI) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-cardiac Troponin I antibody (red; 5A). Nuclei were stained with DAPI (blue; 5A). Dense, filamentous, striated structures were visible in the *rybp*^{+/+} CMCs whilst the appearance was sparsely diffused in the cytoplasm of the *rybp*^{-/-} CMCs (5A). The cTnI signal was one-third of the wild type level in the mutants (5B). The *tnni3* gene was downregulated in the lack of Rybp (5C). (Objective: 60x, zoom: 1.5x; Scale bar: 100 μ m) Deviation: $\pm SD$; Statistical method (t-test type 3): p<0,05 (*p<0,05; **p<0,01; ***p<0,001)

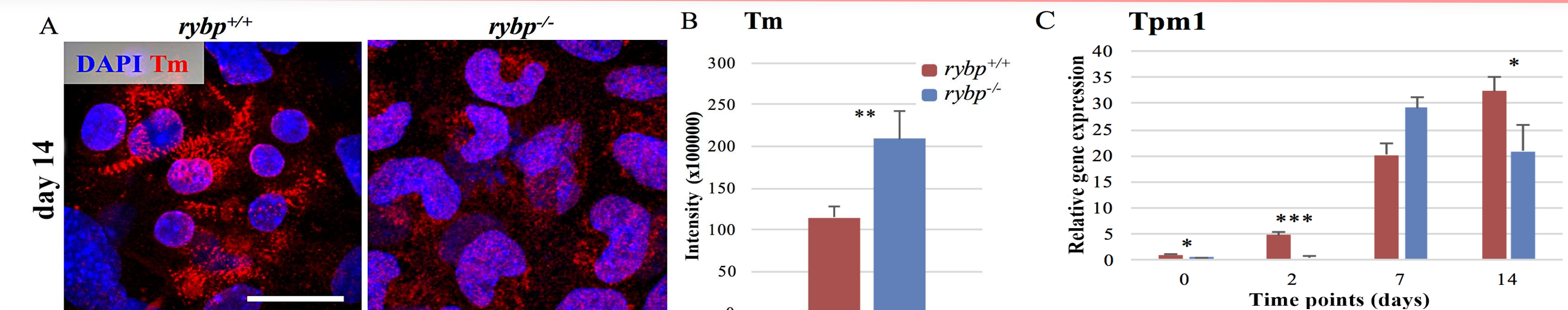


Figure 6: Tropomyosin (Tm) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-Tropomyosin antibody (red; 6A). Nuclei were stained with DAPI (blue; 6A). The sarcomeres were missing in the *rybp*^{-/-} CMCs (6A). There was higher Tm signal in the *rybp*^{-/-} CMCs because of the diffusely distribution of Tm proteins (6B). Cardiac *tpm1* and *tpm4* were downregulated in the *rybp*^{-/-} cells in the terminal phase of cardiac differentiation (day 14) (6C,D). (Objective: 60x, zoom: 3.3x; Scale bar: 20 μ m) Deviation: $\pm SD$; Statistical method (t-test type 3): p<0,05 (*p<0,05; **p<0,01; ***p<0,001)

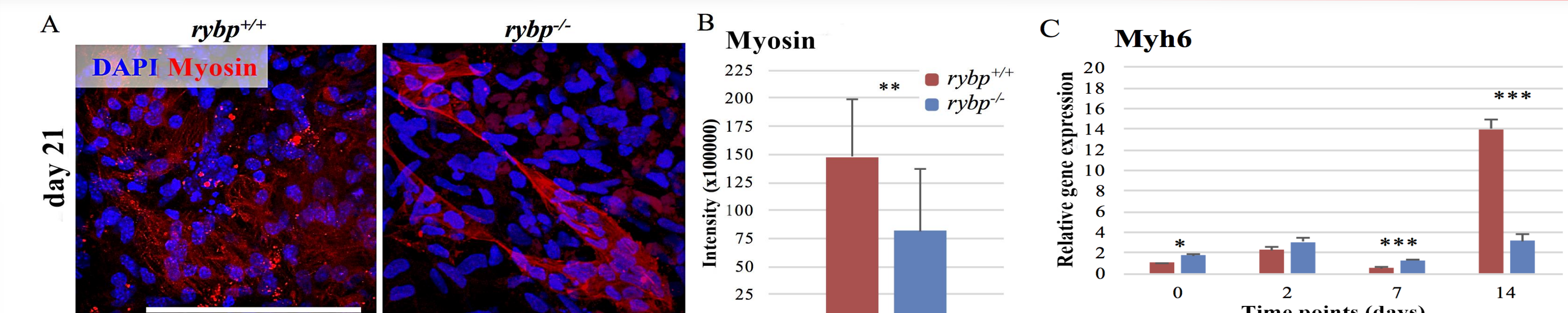


Figure 7: Myosin ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-Myosin-Sarcomere antibody (red; 7A). Nuclei were stained with DAPI (blue; 7A). The *rybp*^{-/-} CMCs did not have any sarcomeres, the filaments were less branching and dense compared to the *rybp*^{+/+} CMCs (7A). The Myosin signal was lower in the *rybp*^{-/-} CMCs (7B). *Myosin heavy chain 6* (*myh6*) and *myosin heavy chain 7* (*myh7*) genes were downregulated in the *rybp*^{-/-} cells (7C,D). (Objective: 60x, zoom: 2.1x; Scale bar: 100 μ m) Deviation: $\pm SD$; Statistical method (t-test type 3): p<0,05 (*p<0,05; **p<0,01; ***p<0,001)

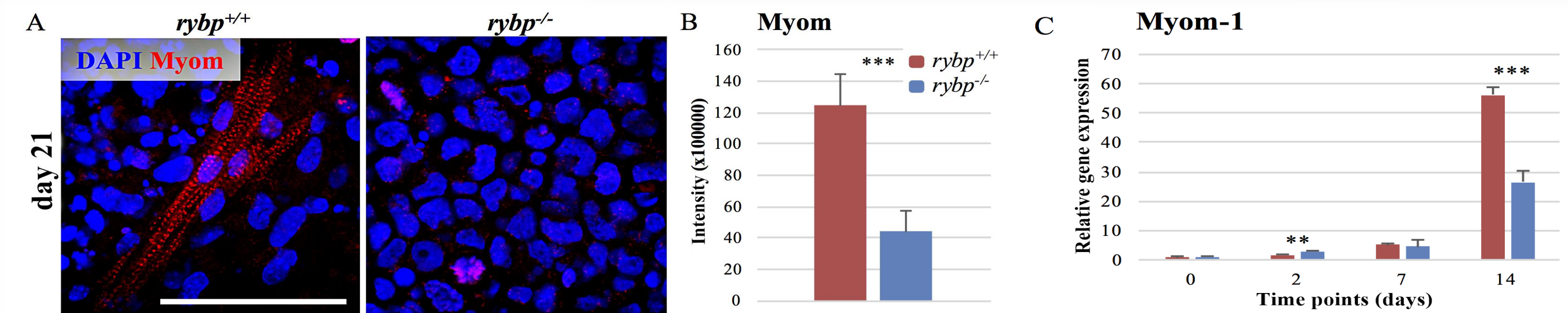


Figure 8: Myomesin (Myom) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-Myomesin antibody (8A). Nuclei were stained with DAPI (blue; 8A). The sarcomeres were missing in the *rybp*^{-/-} CMCs (8A). The intensity of Myom signals was three times higher in the *rybp*^{+/+} CMCs (8B). The cardiac isoform *myom-1* gene expression level was two times higher in the *rybp*^{-/-} cells (8C). (Objective: 60x, zoom: 3x; Scale bar: 50 μ m) Deviation: $\pm SD$; Statistical method (t-test type 3): p<0,05 (*p<0,05; **p<0,01; ***p<0,001)

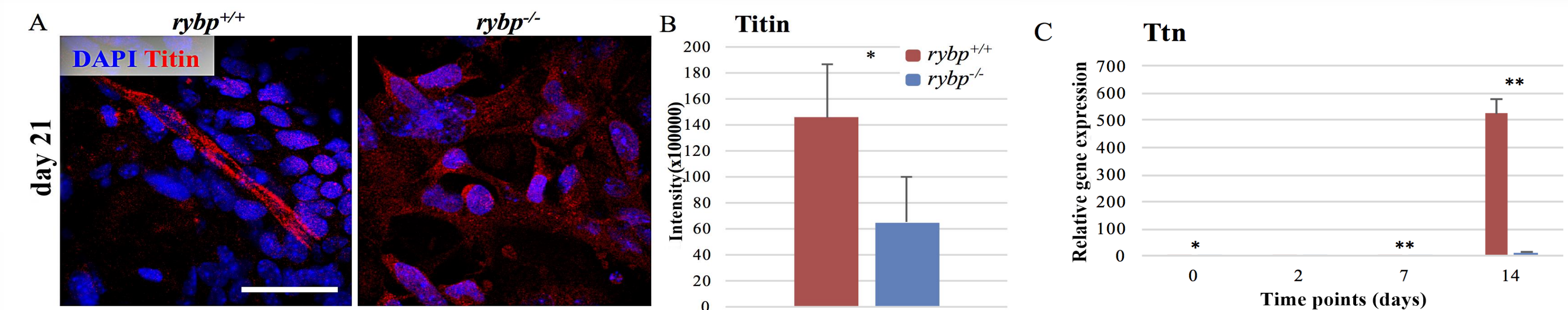


Figure 9: Titin (Ttn) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-Titin antibody (9A). Nuclei were stained with DAPI (blue; 9A). The *rybp*^{-/-} CMCs did not have any sarcomeres (8A). The intensity of Ttn signals was double in the *rybp*^{+/+} CMCs (9B). The *ttn* gene expression level was fifty times higher in the *rybp*^{-/-} cells (9C). (Objective: 60x, zoom: 2.3x; Scale bar: 100 μ m) Deviation: $\pm SD$; Statistical method (t-test type 3): p<0,05 (*p<0,05; **p<0,01; ***p<0,001)

SUMMARY: The thick filament associated proteins (Myosin, Myom, Ttn) and the thin filament associated proteins (cTnT, cTnI, Tm) had altered distribution in the *rybp null* mutant and their gene expression levels (*myh6*, *myh7*, *myom-1*, *ttn*, *tnnt2*, *tnni3*, *tpm1*, *tpm4*) were lower compared to the wild type.

CONCLUSIONS

Sarcomerogenesis is impaired in the absence of Rybp during *in vitro* cardiac differentiation. Rybp is affecting the level of sarcomere structure genes during the process of differentiation and might be important for subcellular localisation/assembly of sarcomeric proteins and subsequent sarcomerogenesis. Rybp may have specific role in the spatiotemporal regulation of cardiac differentiation. Together, our data provides evidence for a previously unrecognized function of Rybp in heart development and sarcomerogenesis. These findings have implications for understanding the development of congenital heart disease and for the further stem cell base therapeutical applications.

- MK Pirity, J Locker, N Schreiber-Agus „Rybp/DEDAF is required for early postimplantation and for central nervous system development” (2005) *Mol Cell Biol*, 25(16): 7193-202
- MK Pirity, et al. „Rybp, a polycomb complex-associated protein, is required for mouse eye development” (2007) *BMC Dev Biol*, 7 p. 39
- O Ujhelly, et al. „Lack of Rybp in Mouse Embryonic Stem Cells Impairs Cardiac Differentiation” (2015) *Stem Cells and Development*, 24(18): 2193-205
- A Nagy, et al. „Derivation of completely cell culture-derived mice from early-passage embryonic stem cells” (1993) *Proc Natl Acad Sci USA*, 90(18): 8424-8
- S Rungtornlert, et al. „Embryoid body formation from embryonic and induced pluripotent stem cells: Benefits of bioreactors” (2009) *World J. Stem Cells*, 1(1): 11-21
- J England and S Loughna "Heavy and light roles: myosin in the morphogenesis of the heart" (2013) *Cell Mol Life Sci*, 70(7): 1221-39
- I Agarkova, JC Perriard "The M-band: an elastic web that crosslinks thick filaments in the center of the sarcomere" (2005) *Trends Cell Biol*, 15(9): 477-85.
- MM LeWinter and HL Granzy „Cardiac titin and heart disease” (2014) *J Cardiovasc Pharmacol*, 63(3): 207-12
- S Ono "Dynamic regulation of sarcomeric actin filaments in striated muscle" (2010) *Cytoskeleton (Hoboken)* 67(11): 677-92
- AS McElhinny, et al. „Nebulin: the nebulous, multifunctional giant of striated muscle" (2003) *Trends Cardiovasc Med* 13(5): 195-201
- G Jagatheesan, S Rajan, DF Wiczorek "Investigations into tropomyosin function using mouse models" (2010) *J Mol Cell Cardiol*, 48(5): 893-8
- AV Gomes, JD Potter, D Szczesna-Cordary "The role of troponins in muscle contraction" (2002) *IUBMB Life*, 54(6): 323-33
- PK Luther "The vertebrate muscle Z-disc: sarcomere anchor for structure and signalling" (2009) *J Muscle Res Cell Motil*, 30(5-6): 171-85.
- H Bär, et al. "The biology of desmin filaments: how do mutations affect their structure, assembly, and organisation?" (2004) *J Struct Biol*, 148(2): 137-52.
- Q Jiao, et al. "Sarcalumenin is essential for maintaining cardiac function during endurance exercise training" (2009) *Am J Physiol Heart Circ Physiol*, 297(2): H576-82.

ACKNOWLEDGEMENTS:

This work was supported by NKTH-OTKA FP7 „Mobility” HUMAN-MB08C-80205