

2014. május 28 - 29.

# STRAUB-NAPOK

MEGHÍVÓ



STRAUB F. BRUNÓ  
BIOKÉMIKUS  
1914 - 1996

MTA Szegedi Biológiai Kutatóközpont

Az Európai Unió Kiválósági Központja

Szeged, Temesvári krt. 62., Nagyelődó



### Why stem cell derived rybp deficient cardiomyocytes are not contractible?

Viktória Szabó, Flóra Vajda and Melinda Pirity  
(Institute of Genetics, BRC HAS, Szeged)

### Dosage dependent effect of Rybp on the retinoic acid mediated signalling pathway

Gergő Kovács, Árpád Varga, Viktória Szabó and Melinda Pirity  
(Institute of Genetics, BRC HAS, Szeged)

### In situ study of embryonic enhancers flanking a chromatin silencer in *Drosophila*

Viktória Kiss and László Sipos  
(Institute of Genetics, BRC HAS, Szeged)

### The role of Spartan (c1orf124) in DNA damage tolerance

Orsolya Berczeli, Mónika Mórocz, Szilvia Juhász, Péter Burkovics and Lajos Haracska  
(Institute of Genetics, BRC HAS, Szeged)

### Structural and functional analysis of the sessile hematopoietic tissue in *Drosophila melanogaster*

Viktor Honti\*, Gábor Csordás\*, Gergely I. B. Varga\*, Ferenc Jankovics, Éva Kurucz and István Andó  
(<sup>1</sup>Institute of Genetics, BRC HAS, Szeged;  
\*These authors contributed equally to the work.)

### In vivo immunostaining of hemocyte compartments in *Drosophila* for live imaging

Gábor Csordás\*, Gergely I. B. Varga\*, Viktor Honti\*, Ferenc Jankovics, Éva Kurucz and István Andó  
(Institute of Genetics, BRC HAS, Szeged;  
\*These authors contributed equally to the work.)

### Spring, summer, autumn, winter: changes in honey bee hemocytes' number and in the rate of different cell types through the year

Erika Gábor<sup>1</sup>, Tibor Török<sup>2</sup>, János Zsámboki<sup>1</sup>, Gyöngyi Cinege<sup>1</sup>, Gábor Csordás<sup>1</sup>, Viktor Honti<sup>1</sup>, Éva Kurucz<sup>1</sup> and István Andó<sup>1</sup>  
(<sup>1</sup>Institute of Genetics, BRC HAS, Szeged;  
<sup>2</sup>Department of Genetics, University of Szeged, Szeged)

### Functional analysis of Vajk proteins in *Drosophila melanogaster*

Gyöngyi Cinege, János Zsámboki, Gábor Csordás, Viktor Honti, István Andó and Éva Kurucz  
(Institute of Genetics, BRC HAS, Szeged)

### A novel septic injury method for the identification of factors involved in host-pathogen interactions in *Drosophila melanogaster*

Beáta Kari, János Zsámboki, Viktor Honti, Gábor Csordás, Róbert Márkus, István Andó and Éva Kurucz  
(Institute of Genetics, BRC HAS, Szeged)



# Why Stem Cell Derived *rybp* Deficient Cardiomyocytes Are Not Contractible?

Viktória Szabó, Flóra Vajda and Melinda Pirity  
Institute of Genetics, BRC-HAS, Szeged

## RATIONALE

Congenital heart defects are heart problems that develop before birth. They can occur in the heart's chambers, valves or blood vessels. The defects of heart development arise at an early stage because of the anomaly of molecular pathways governing embryonic heart development. 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 embryonic development [1]. Since *rybp* null embryos die at implantation [1] the *in vivo* function of this gene during later stage organ development cannot be studied.

## OBJECTIVE

The objective of this study was to determine to role of Rybp in murine cardiac differentiation in Embryonic Stem (ES) cells based model system [2]. We utilized *rybp* null ES cells (Figure 1) [4] and differentiated them towards cardiac lineages *in vitro* (Figure 2 and Materials and methods) in order to unveil a novel role of *rybp* in heart development.

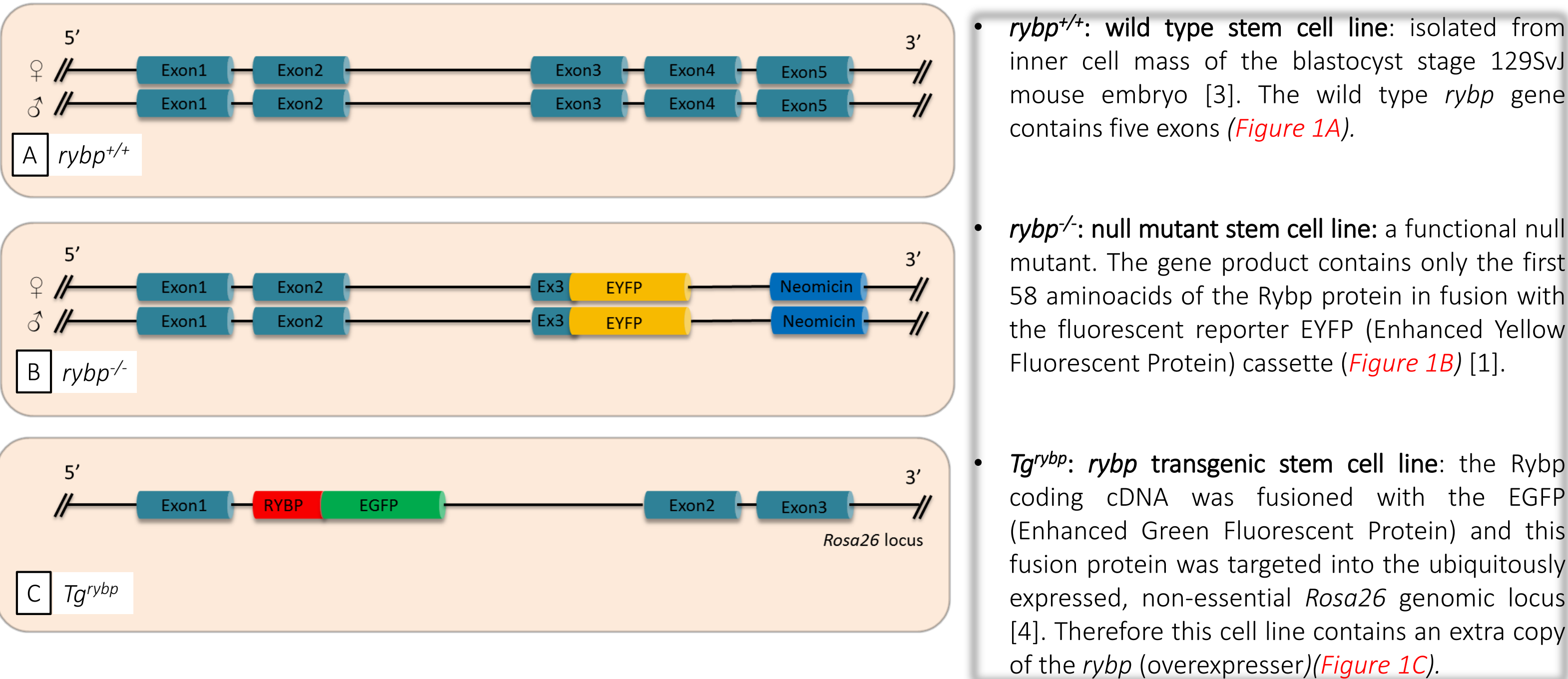


Figure 1: Stem Cell Lines

## MATERIALS AND METHODS

### *In vitro* cardiac differentiation:

Cardiomyocytes differentiated *in vitro* from ES cells by using Hanging Drop (HD) method [5]. In brief: Embryonic Bodies (EBs) were formed using 800 cells per drop (4x10<sup>5</sup> cells/ml). Individual EBs were placed into wells of a 24-well plate containing 0.1 % gelatin coated cover-slips on the second day (d2). The differentiation medium was changed every second day. Cardiomyocytes were grown for 18 days and observed for beating daily, under a phase-contrast microscope. Sampling time points were at day 0, 7 and 14 for further gene expression studies (see below) and at day 18 for immunohistochemistry (see below) (Figure 2).

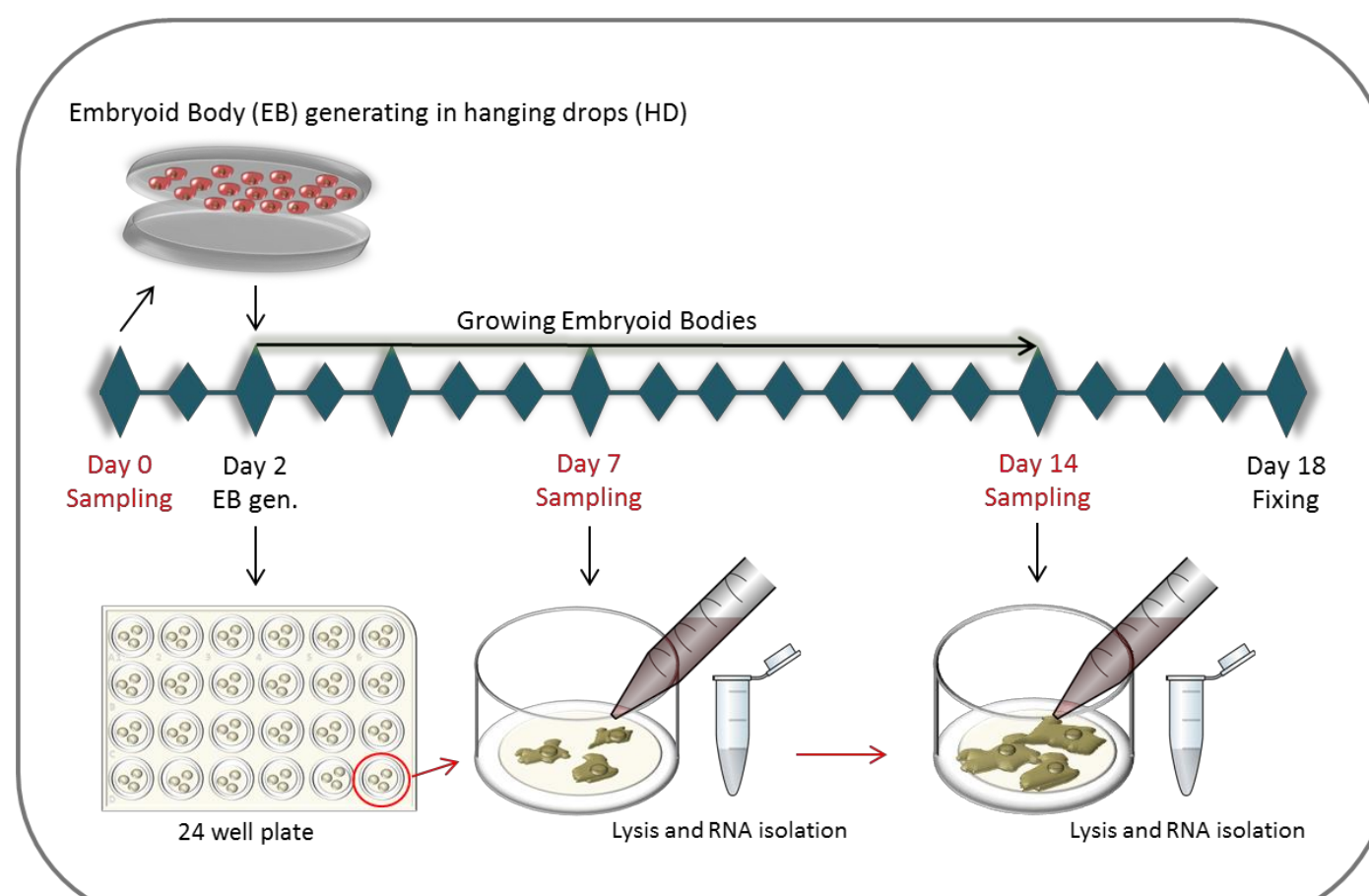


Figure 2: Generating EBs by the „Hanging Drops” method and cardiac differentiation

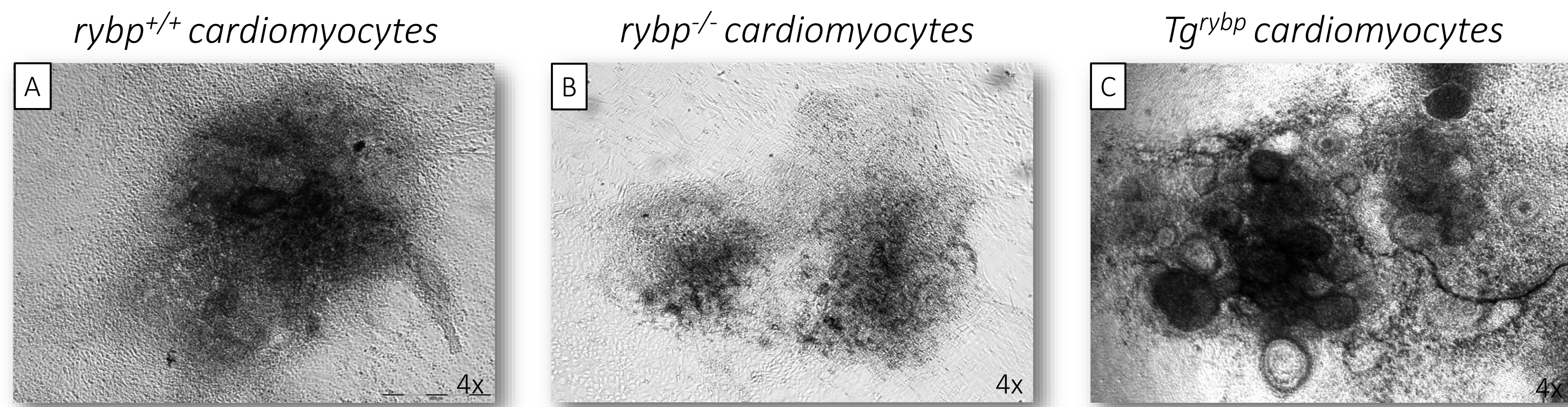
### Quantitative Real-Time PCR (qRT-PCR):

RNA was isolated from the cardiomyocytes colonies using Qiagen Rneasy® Plus Mini Kit (Cat.:74143) according to the manufacturer's protocol. RNA was translated to cDNA with Reverse Transcription Kit (Applied Biosystems). We used the cDNA in qRT-PCR reaction to determine gene expression changes (*rybp*, Oct4, Zac1, cTnT). Reaction contained 5.4µl cDNA + 0.6µl primer mix + 6µl Applied Biosystems Sybr Select Master Mix for CFX. Reactions were run in Bioer LineGeneK Real-time PCR System machine (Bioer, China). Relative gene expression changes were quantified using the ΔΔCt method. The threshold cycle (Ct) values for each gene were normalized to expression level of Hprt and Gapdh. To calculate fold change, the values compared to undifferentiated samples (d0, *rybp*<sup>+/+</sup>) (Figure 4).

### Immunohistochemistry (IHC) of cardiomyocytes:

Cardiomyocytes were washed with phosphate-buffered saline (PBS) and fixed with 4% (v/v) paraformaldehyde (PFA) for 20 min at room temperature (RT). After permeabilization in 0.2% Triton X-100 in PBS for 20 min at RT; cells were blocked in 3% Bovine Serum Albumin (BSA) in PBS for 1h at RT. Then sequentially incubated with the following primary antibodies in 3% BSA overnight at 4°C: Rybp (Rbx DEDAF; Millipore, Cat. AB3637, 1:1000), Oct4 (Octamer-binding transcription factor 4, Mouse Oct-3/4 (C-10); Santa Cruz, Cat. Sc-5279, 1:100), cardiac Troponin T (Anti-Cardiac Troponin T; ABCAM, Cat. ab8295, 1:2000). The cultures were washed 5 times (10 min) with PBS and then blocked in BSA for 1h at RT. Incubated with fluorescent-labeled secondary antibodies (Alexa Fluor 647 G-A-R IgG, Cat. A-21244; Alexa Fluor 546 G-A-M IgG, Cat. A-11030; Alexa Fluor 647 D-A-M IgG, Cat. A-31571) and rhodamine-phalloidin for 1h and washed 3x in PBS. The cells were rinsed in 4',6'-diamidino-2-phenylindole (DAPI; Vector Laboratories, Cat. H-1200) for 20 min, 2x washed in PBS and mounted in 50% glycerol. The images were obtained using Olympus Confocal microscope and Olympus CellIR Imaging microscope (Figure 5,6,7).

## RESULTS



### *rybp*<sup>+/+</sup> cardiomyocytes:

The cardiomyocytes derived from wild type (*rybp*<sup>+/+</sup>) ES cell line grows in multiple layers and shows contractile activity (Figure 3A).

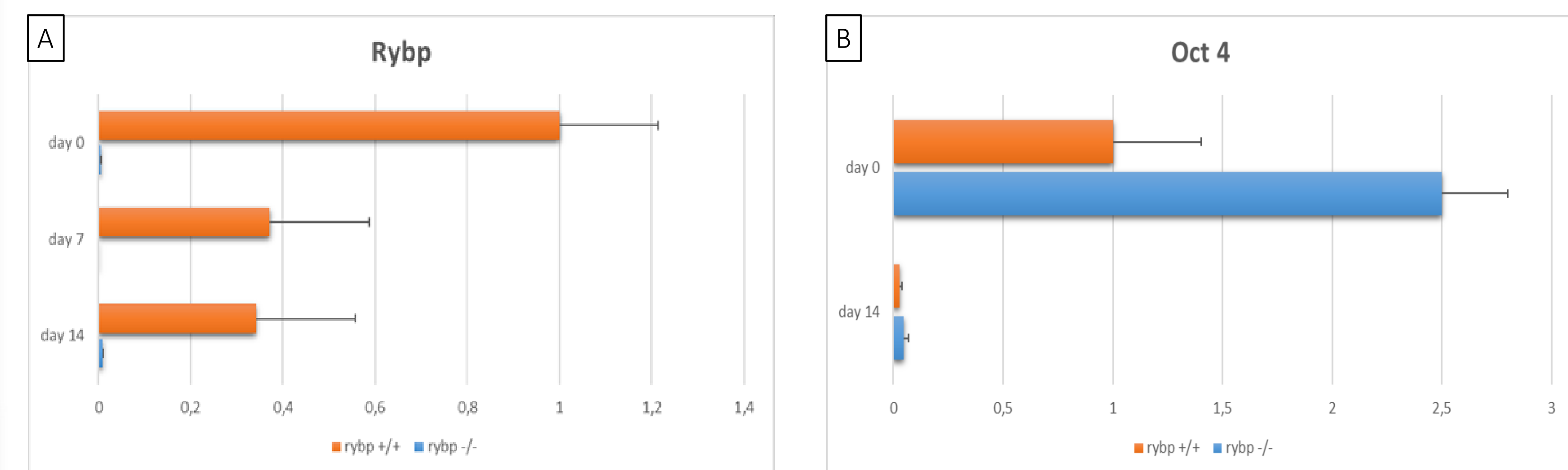
### *rybp*<sup>-/-</sup> cardiomyocytes:

The cardiomyocytes derived from the mutant (*rybp*<sup>-/-</sup>) ES cell line exhibits similar gross morphology and shows contractile activity (Figure 3B).

### *Tgrybp* cardiomyocytes:

The cardiomyocytes derived from the *Tgrybp* ES cell line exhibits similar gross morphology and contractibility like the wild type (Figure 3C).

Figure 3: Microscopic analysis of the cardiac myocytes (Olympus CellIR Imaging, IX81 Microscope)

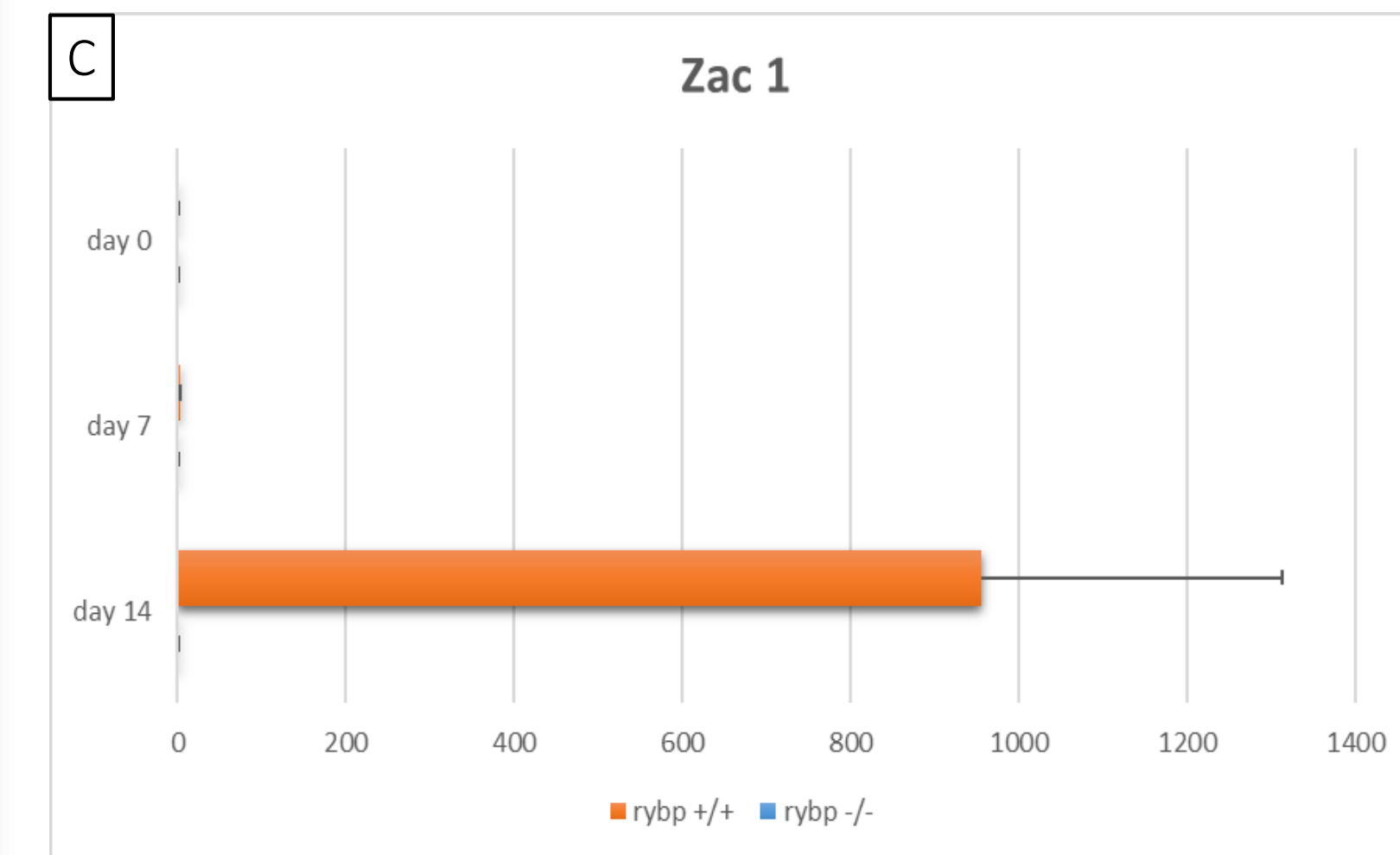


### Expression of Rybp during *in vitro* cardiac differentiation.

In the wild type cells expression of *rybp* decreased by the progress of cardiac differentiation and it is not detectable in the *rybp* null mutants (Figure 4A). (*Rybp*: Ring1 and YY1 binding protein - UniGene Mm.321633; MGI:1929059)

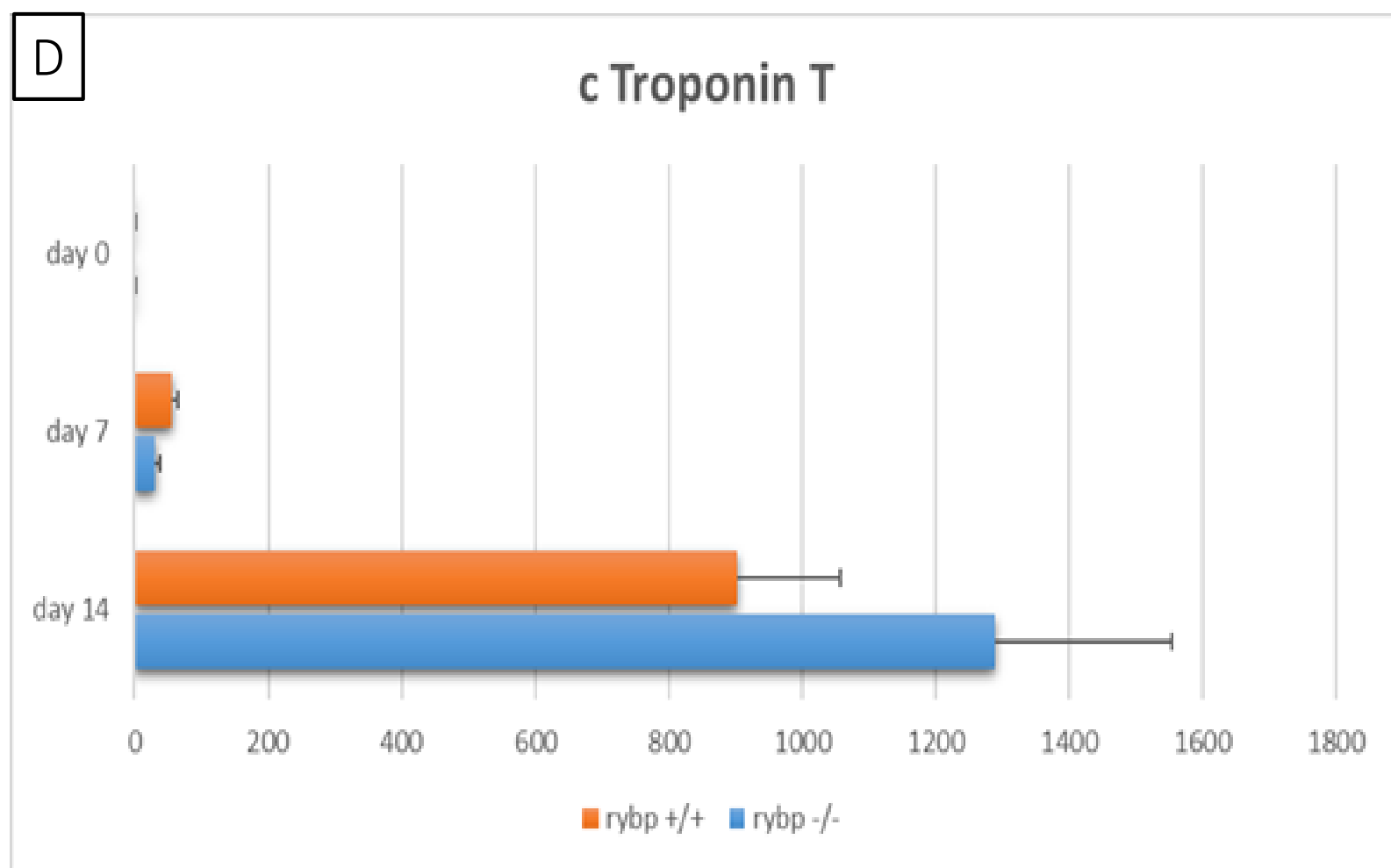
### Expression of Oct4 during *in vitro* cardiac differentiation.

Pluripotency marker Oct4 was expressed in proliferating ES cells (day 0). By differentiation, the level of Oct4 expression was decreased and by d14, as it is expected, it was not detectable any longer in the cell lines (Figure 4B). (*Oct4*: Octamer binding factor 4 - Unigene Mm.17031; MGI:101893) [6]



### Expression of Zac1 during *in vitro* cardiac differentiation.

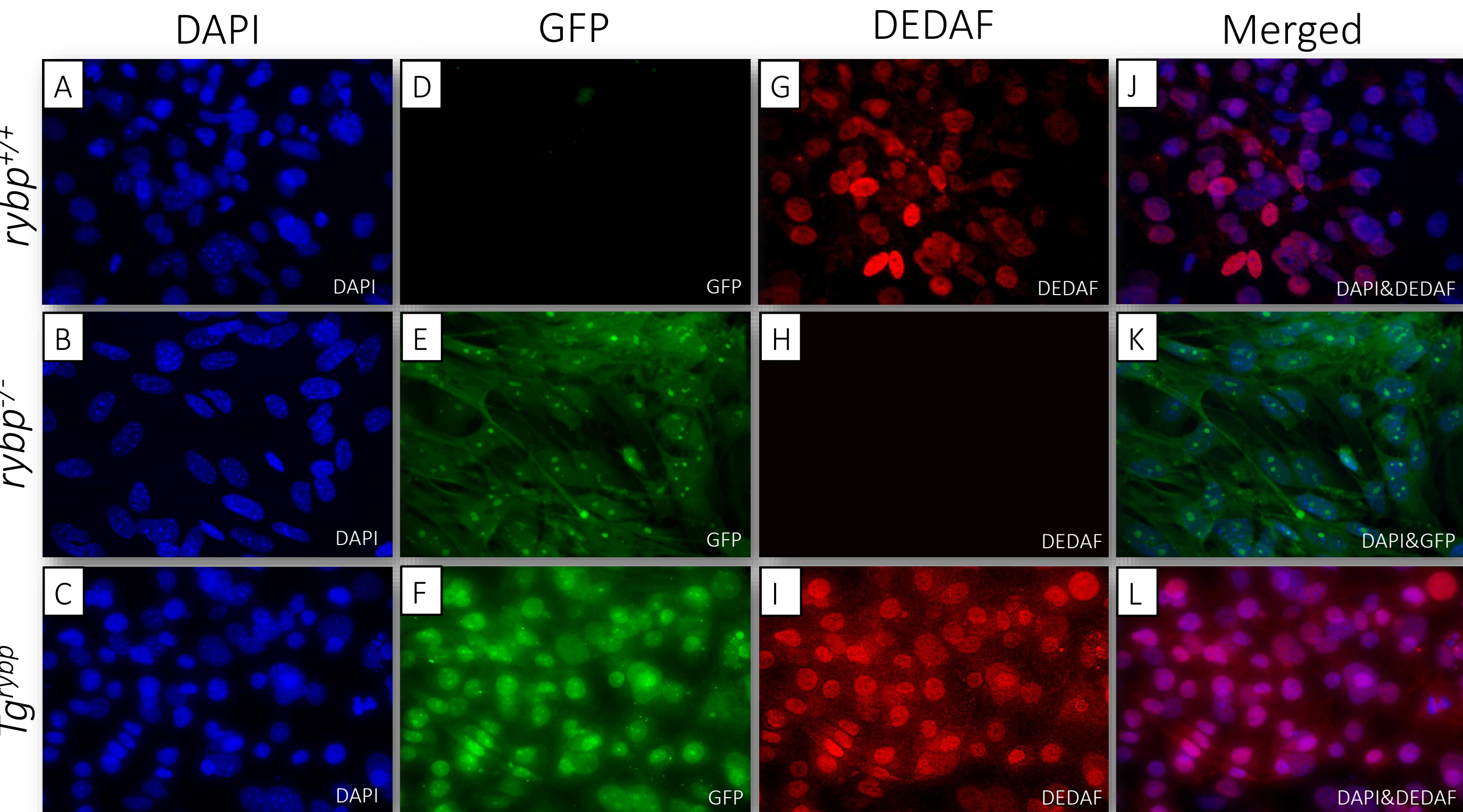
The expression of the cardiac transcription factor Zac1 was induced in the wild type ES cells during cardiac differentiation. *Rybp* null mutants exhibit deficiency in Zac1 expression (Figure 4C). (*Zac1*: Zinc finger protein regulating apoptosis and cell-cycle arrest - UniGene Mm287857; MGI:1100874) [7]



### Expression of cTnT during *in vitro* cardiac differentiation.

Cardiac Troponin T (cTnT) is a key marker of cardiac specification. Its expression was induced by d7 (30x) and d14 (900x) in the wild type cells. Expression profile of cTnT was similar in both cell lines (Figure 4D). (*cTnT*: cardiac troponin T - UniGene Mm.247470; MGI:104597) [8]

Figure 4: qRT-PCR showing relative gene expression levels during *in vitro* cardiac differentiation of the *rybp*<sup>+/+</sup> and *rybp*<sup>-/-</sup> cardiomyocytes



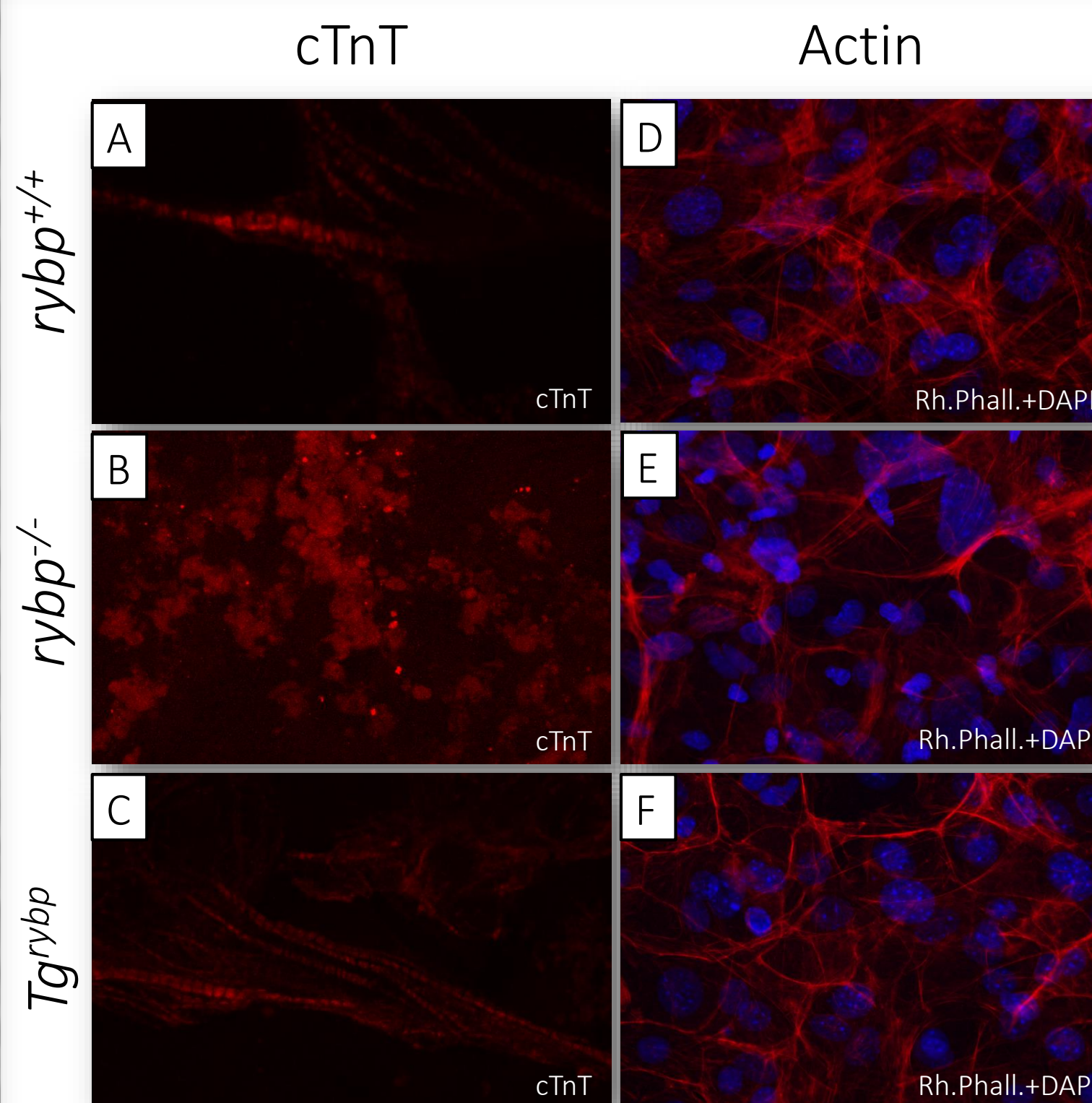
### Figure 5: DEDAF immunostaining analysis of cardiomyocytes derived from *rybp*<sup>+/+</sup>, *rybp*<sup>-/-</sup> and *Tgrybp* ES cells

Cardiomyocytes were stained with antibody for Rybp (red; 5G,H,I). Nuclei were stained with DAPI (blue; 5A,B,C). The subcellular localisation of the knock-in EYFP construct in the *rybp*<sup>-/-</sup> cells is cytoplasmic (5E) and in the *Tgrybp* cells EGFP is nucleolar (5F) due to the presence of Rybp in fusion with EGFP. The wild type cells express endogenous *Rybp* which is also nucleolar (5G). As expected, Rybp is not detectable in the *rybp* null mutants (5H) and overexpressed in the *Tgrybp* cardiomyocytes (5I, strong signal compare to the wild type). Notably, Rybp expression is non-uniform in the *rybp*<sup>+/+</sup> and *Tgrybp* cardiomyocytes (5J,L).

### Materials:

- DAPI; Vector Laboratories, Cat. H-1200
- Rbx DEDAF; Millipore, Cat. AB3637
- Alexa Fluor 647 G-A-R IgG, Cat. A-21244

Objective: 40x, Olympus CellIR Imaging m.



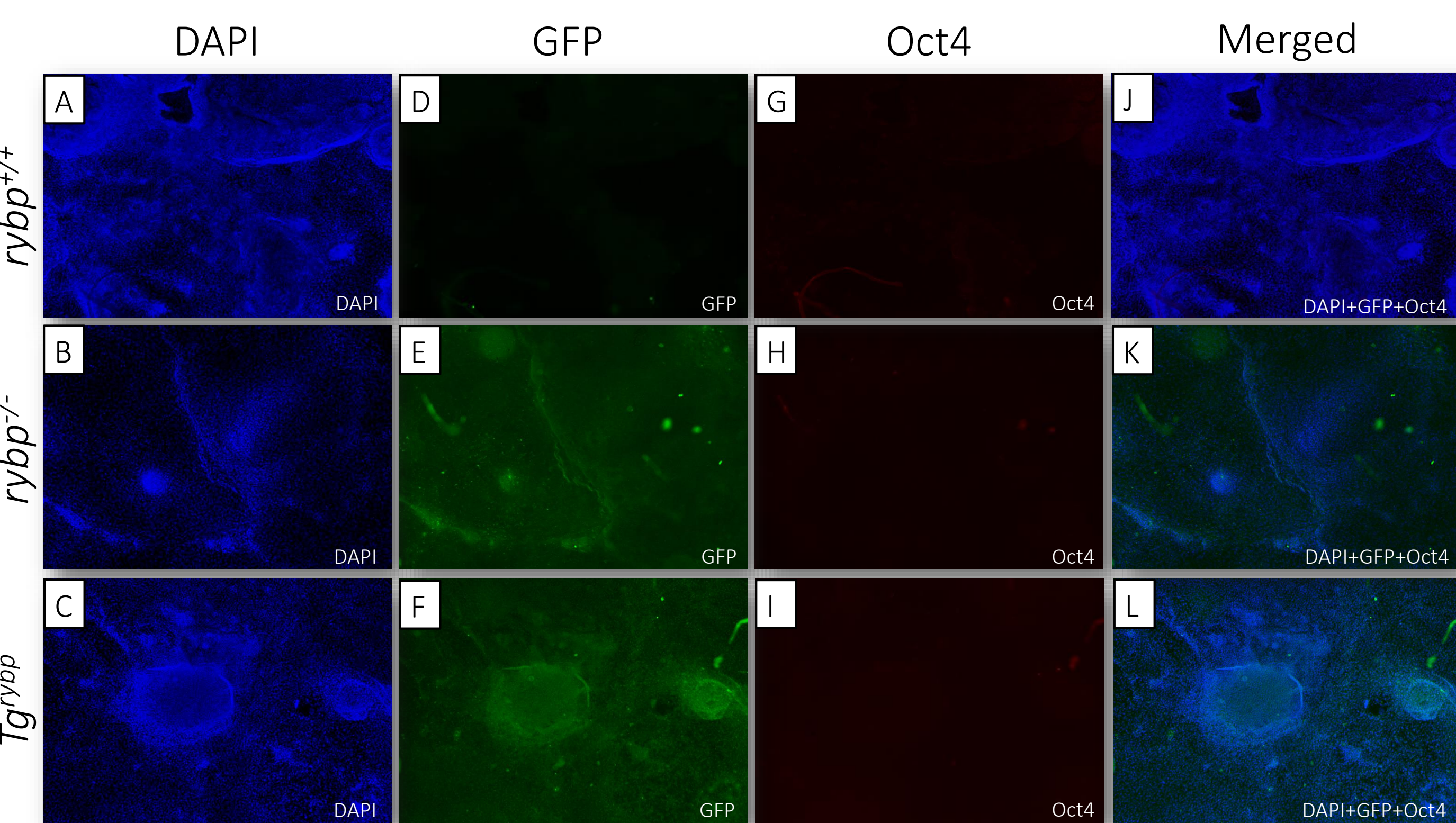
### Figure 6: Cardiac differentiation potential of EBs derived from *rybp*<sup>+/+</sup>, *rybp*<sup>-/-</sup> and *Tgrybp* ES cells

Cardiomyocytes were stained with the antibody against cTnT (red; 6A,B,C) and with rhodamine-phalloidin for actin (red; 6D,E,F). Nuclei were stained with DAPI (blue; 6D,E,F). There is normal sarcomere formation in the *rybp*<sup>+/+</sup> and *Tgrybp* cells (6A,C). cTnT were not assembled into sarcomere in the *rybp*<sup>-/-</sup> cardiomyocytes (6B). Rhodamine-phalloidin staining was applied to visualize distribution of F-actin. There is normal actin-filament structure in the *rybp*<sup>+/+</sup> and *Tgrybp* cells (6D,E,F).

### Materials:

- DAPI; Vector Laboratories, Cat. H-1200
- Anti-Cardiac Troponin T; ABCAM, Cat. ab8295
- Alexa Fluor 546 G-A-M IgG, Cat. A-11030
- Rhodamine-phalloidin

Actin; Objective: 40x, Olympus CellIR Imaging m. cTnT; Objective: 40x, Olympus Confocal m.



### Figure 7: Immunostaining of cardiomyocytes derived from *rybp*<sup>+/+</sup>, *rybp*<sup>-/-</sup> and *Tgrybp* ES cells for the pluripotency marker Oct4

Cardiomyocytes were stained with antibody for Oct4 (7G,H,I). Nuclei were stained with DAPI (blue; 7A,B,C).

Immunostaining for Oct4 confirmed that during *in vitro* cardiac differentiation the pluripotency of the cells was lost. (7G,H,I).

### Materials:

- DAPI; Vector Laboratories, Cat. H-1200
- Mouse Oct-3/4 (C-10); Santa Cruz, Cat. Sc-5279
- Alexa Fluor 647 D-A-M IgG, Cat. A-31571

Objective: 4x, Olympus CellIR Imaging m.

The assembly/disassembly of multiprotein complexes during sarcomerogenesis follows ordered pathways, which are regulated on the transcriptional, translational and posttranslational level. Zac1 is an important component in this process as colocalizes with  $\alpha$ -actinin, the member of Z-disk (Figure 8A). We hypothesize that Rybp is an upstream regulator of Zac1 and regulates Zac1 during sarcomerogenesis (Figure 8C). When *rybp* is not present (*rybp*<sup>-/-</sup> cardiomyocytes) Zac1 is not expressed and sarcomerogenesis is impaired (Figure 8B). Most likely Rybp acts in this process as a part of a multimeric complex or heterodimers regulating sarcomerogenesis.

## CONCLUSION

We found that in the absence of Rybp cells show defect in cardiac differentiation. Our study revealed that lack of Rybp is not affecting the level of cTnT during *in vitro* cardiac differentiation, rather then may be important for the subcellular localisation/assembly of cTnT and subsequent sarcomerogenesis (Figure 8). Gene expression analysis uncovered that Zac1 is regulated by Rybp during *in vitro* cardiac differentiation. Currently gene expression profile analysis is being performed to reveal the genetic network Rybp may affect. These findings are likely to place RYBP in processes of cardiac differentiation and have implications for the understanding of heart diseases. Together our data provide evidence for a previously unrecognized function of Rybp in heart development.

## REFERENCES

- [1] MK Pirity, WJ Wang, LV Wolf, ER Tamir, N Schreiber, Agas, A Cvelil, Rybp, a polycomb complex-associated protein, is required for mouse eye development. BMC Dev Biol. 2007; doi:10.1186/1471-213X-7-39
- [2] CE Murry, C Keller, Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell. 2008; doi:10.1016/j.cell.2008.02.008
- [3] A Nagy, J Rosent, R Nagy, W Abanow-Newerly, JC Rhee, Derivation of completely cell culture-derived mice from early passage embryonic stem cells. Proc. Natl. Acad. Sci. USA. 1993; doi:10.1073/pnas.90.12.5769
- [4] MK Pirity, J Losler, N Schreiber-Agus, Rybp/SCDF is required for Early Postimplantation and for Central Nervous System Development. Mol Cell Biol. 2005; doi:10.1128/MCB.25.16.7159-7172.2005
- [5] S Rungtornart, M Tachikunpha, MK Pirity, A Dronyes, Embryoid body formation from embryonic and induced pluripotent stem cells: Benefits of bioactors? World J. Stem Cells. 2009; doi:10.4252/wjsc.v1.i1.11
- [6] K Takahashi, S Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; doi:10.1016/j.cell.2006.03.024
- [7] S Naka, T Ohtsuka, K Shimizu, Y Ohno, T Tagawa, S Ito, T Egashira, T Sato, H Tsukamoto, T Hoshikawa, K Kaneda, M Murata, T Hattori, S Makino, M Sano, S Ogawa, ZW Pfaff, RP Harvey, K Fukuda, Zac1 is an essential transcription factor for cardiac morphogenesis. Circ Res. 2010; doi:10.1161/CIRCRESAHA.109.214330
- [8] E Kishi, S Morimoto, K Minakami, Y Miyeno, K Hashizume, M Ohta, DY Zhao, QW Lu, Y Shibata, Targeted disruption of the cardiac troponin T gene causes sarcomere disassembly and defects in heartbeat within the early mouse embryo. Dev Biol. 2008; doi:10.1016/j.ydbio.2008.07.007

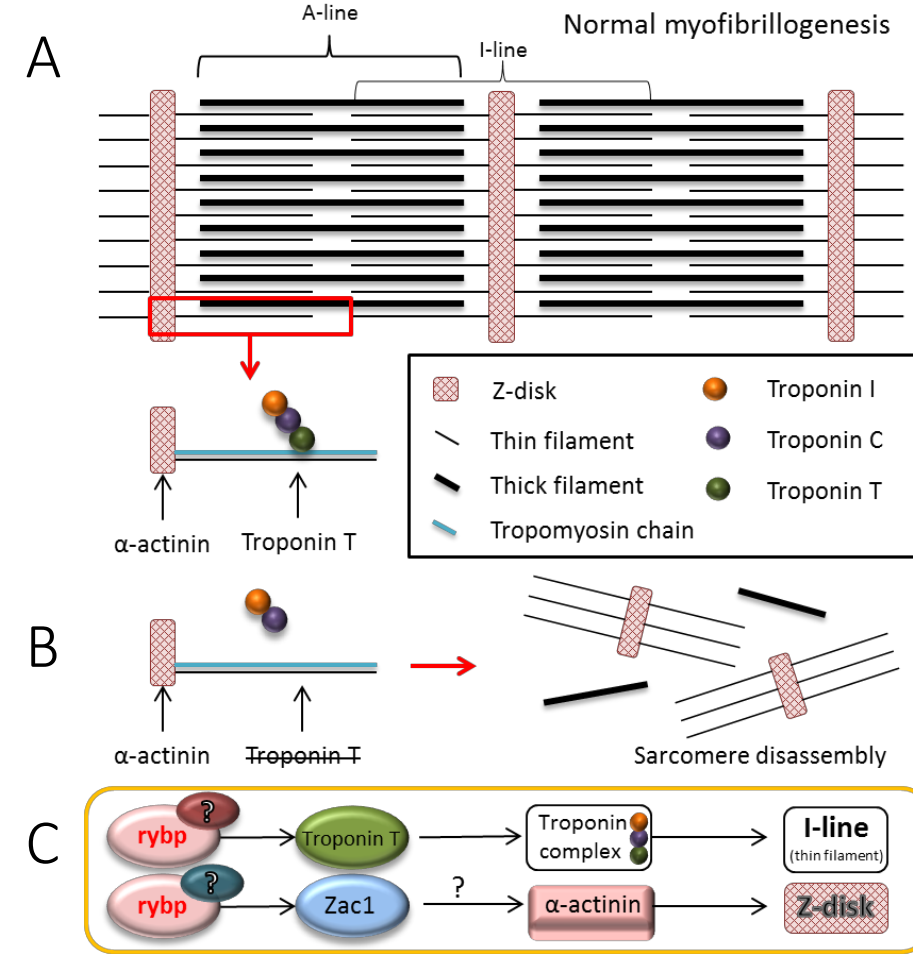


Figure 8: Schematic model for "Why *rybp* Deficient Cardiomyocytes Are Not Contractible?"