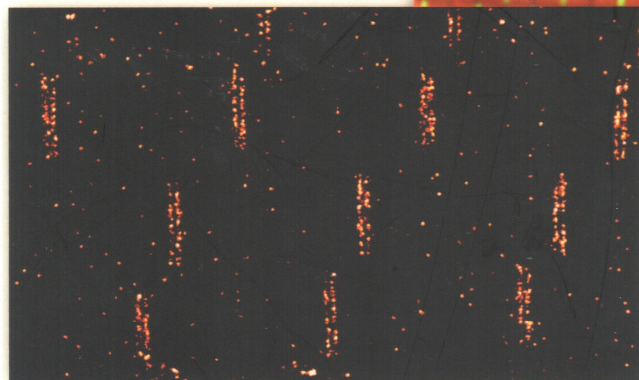
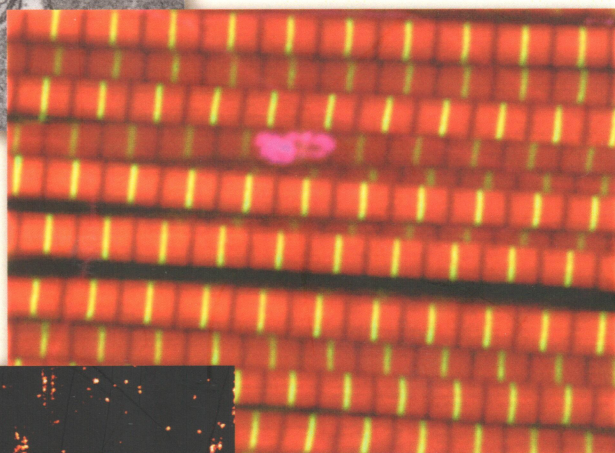
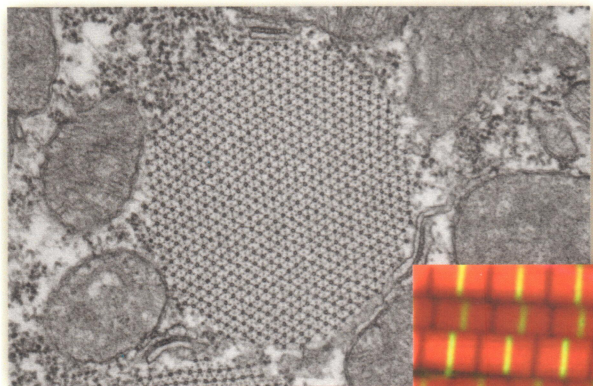
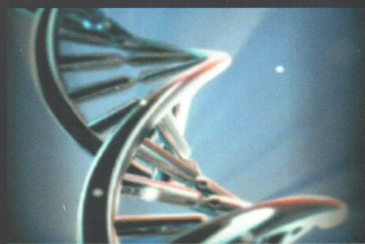


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consequent differentiation. Previously, we have found that reduced level of Rybp in mice caused neural tube defects, which are the second most common congenital anomaly in humans. We have previously described that Rybp depletion suppressed *in vitro* neural differentiation since *rybp null* mutant (*rybp*^{-/-}) mouse embryonic stem (ES) cells formed less terminally differentiated neural cells from existing progenitors. We also found an increased rate of neural progenitor formation in the *rybp null* mutants, which coincided with the elevated mRNA level of RA-regulated transcription factor, Pax6.

In current work, we used a stem cell-based *in vitro* differentiation method to study the role of Rybp in RA signalling: wild-type (*rybp*^{+/+}) and *rybp null* mutant (*rybp*^{-/-}) mouse ES cells were differentiated into neural lineages through embryoid body (EB) formation, where neural differentiation was induced by RA treatment. Samples were taken on day 0 (d0), d3, d7, d10 and d14 and the relative gene expression changes of the RA pathway members were examined with quantitative real-time polymerase chain reaction (qRT-PCR). We also examined the morphology of the EBs and the changes of RA-regulated Pax6 protein distribution in the presence and/or absence of RA.

We have found that (i) the expression of the RA-regulated genes (e.g. Pax6, Stra6) and the complex members, which responsible for the transfer of cytosolic RA into nucleus (e.g. Crabp) were increased in absence of Rybp and (ii) pathway members facilitating RA degradation (e.g. Cyp26) were highly enhanced. Based on these findings we postulate that the *rybp null* mutant cells have an elevated level of the RA pool and the RA metabolism is altered in the mutants.

In summary, lack of Rybp caused the alterations of RA pathway which resulted modified expression of RA target genes. Since majority of RA target genes play important part in differentiation processes, including neural differentiation, we postulate that alteration of RA pathway may play a major role in the neural differentiation defects caused by the absence of Rybp.

This work was supported by GINOP-2.3.2-15-2016-00001 and GINOP-2.3.2-15-2016-00039.

Keywords: Rybp, stem cells, retinoic acid pathway, *in vitro* neural differentiation

P-143

Rybp NULL MUTANT STEM CELLS DO NOT FORM FUNCTIONAL SARCOMERES DURING *IN VITRO* CARDIAC DIFFERENTIATION

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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 Polycomb Group (PcG) member, Rybp (Ring1- and Yy1-Binding Protein; also known as DEDAF (Death Effector Domain Associated Factor), UniGene Mm.321633; MGI:1929059), which is transcriptional regulator with essential role in early embryonic development. We have previously showed that lack of Rybp in mouse embryonic stem (ES) cells impairs cardiac differentiation resulting cardiomyocytes (CMCs) with no contractile activity. Since contractility highly depends on the presence of intact sarcomeres, the objective of this work was to characterise the *rybp null* mutant CMCs in respect to their sarcomere remodeling abilities. We have used an embryonic stem (ES) cell based model system where wild-type (*rybp*^{+/+}) and *rybp null* mutant (*rybp*^{-/-}) mouse ES cells were differentiated to CMCs, then collected samples at different timepoints of differentiation for measuring the gene expression changes of sarcomeric components by qRT-PCR analysis and examining the spatiotemporal localization of sarcomeric components by immunocytochemistry (ICC). We examined sarcomere components, such as thick filament associated proteins (myosin, myomesin, titin) and thin filament associated proteins (troponin T, troponin I, tropomyosin). Our results indicate that (1) the cardiac sarcomere structure was not properly formed in the mutants, (2) the expressions of the corresponding genes decreased and (3) their localization were aberrant. In summary, we present evidence the first time that Rybp is a key factor of the heart contractility and Rybp may be a new player of the development of congenital heart diseases.

Acknowledgements: This work was supported by GINOP-2.3.2-15-2016-00001 and GINOP-2.3.2-15-2016-00039.

Keywords: *rybp, polycomb, cardiac differentiation, sarcomere*

P-144

THE VASOACTIVE EFFECTS OF STEM CELL-DERIVED ENDOTHELIAL CELLS IN ISOLATED RAT AORTIC RING MODEL

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Human embryonal (hESC-EC) and induced pluripotent stem cell-derived endothelial cells (hiPSC-EC) may play roles in the regenerative therapy of ischemic cardiovascular disease and can be well applied for drug development assays. Functional characterisation of hPSC-ECs is required for their safe application to clinical use. Indeed, ECs are involved in inflammatory, vasoactive, and haemostatic processes.

We thought to examine therefore the vasoactive effect of hPSC-EC cultures in isolated rat aortic ring model. Human umbilical vein endothelial cells (HUVEC) were used as control in the experiments. The aortic vessels were isolated and prepared from standard housed adult



Rybp null mutant stem cells do not form functional sarcomeres during *in vitro* cardiac differentiation

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INTRODUCTION

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 differentiation plays a central role in reduced cardiac pump function in heart failure. Our laboratory focused on the early onset of cardiac development by utilizing embryonic stem cell (ES) based *in vitro* cardiac differentiation system. We have previously shown that lack of the Poliovirus Group (PVG) member, Rybp (Rybp^{-/-}) and 121 Binding Protein, also known as DIDA4 (Dish Effector Domain Associated Factor), (UniProt M32163), MG1-92909) in ES cells impairs cardiac differentiation resulting cardiomyocytes (CMCs) with no contractile activity [1]. Since contractility highly depends on the presence of intact sarcomeres, the objective of this work was to characterize the *rybp* null mutant CMCs in respect to their sarcomeric remodeling abilities. We have differentiated wild-type (*rybp*^{+/+}) and *rybp* null mutant (*rybp*^{-/-}) mouse ES cells to CMCs and collected samples at different timepoints of differentiation for measuring the gene expression changes of sarcomeric components by qRT-PCR analysis and examining the spatiotemporal localization of sarcomeric components by immunocytochemistry (ICC). We examined sarcomeric components such as thick filament associated proteins (myosin, myosin, titin) and thin filament associated proteins (alpha cardiac actin, tropomyosin I, tropomyosin T, troponin). Our results indicated that (1) the cardiac sarcomeric structure was not properly formed in the *rybp*^{-/-} mutants, (2) the expression of the corresponding genes decreased and (3) their localization were aberrant. In summary, we present evidence the first time that Rybp is a key factor of the heart contractility and Rybp may be a new player of the development of congenital heart diseases.

STEM CELL LINES

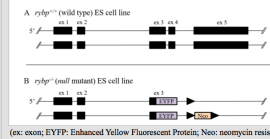


Figure 1: *rybp*^{+/+} and *rybp*^{-/-} stem cell lines

The wild type stem cell line (*rybp*^{+/+}) was isolated from the inner cell mass of the blastocyst stage 129SvEv mouse embryos. The wild type *rybp* gene contains five coding exons (1-5) [2].

The *rybp*^{-/-} cell line harbors a functional null mutant. The gene product contains only the first 58 aminoacids of the Rybp protein in fusion with the fluorescent reporter EYFP (Enhanced Yellow Fluorescent Protein) and with the neomycin resistance gene cassette (1/9) [3] resulting a functional null mutant.

Thin filament associated proteins (C)

Myosin (pink) is a motor protein, it helps to relay energy and generating force [4]. Myosin (yellow) is a myosin anchoring protein in the M-line and it connects with the thin [5]. Titin (blue) is a molecular spring in the plastic elasticity of the striated muscle and helps for myosin to survive strain during contraction [6].

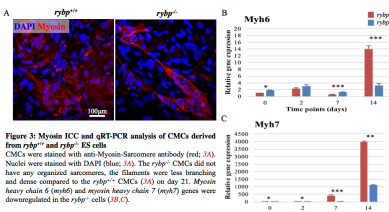


Figure 3: Myosin ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-Myosin-Sarcomeric antibody (red, 3A). Nuclei were stained with DAPI (blue, 3A). The *rybp*^{-/-} CMCs did not have any organized sarcomeres, the filaments were less branching and dense compared to the *rybp*^{+/+} CMCs (3A) on day 21. Myosin heavy chain 6 (*myh6*) and myosin heavy chain 7 (*myh7*) genes were downregulated in the *rybp*^{-/-} cells (3B,C).

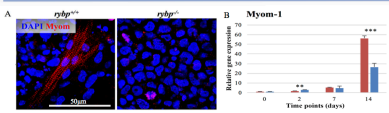


Figure 4: Myosin (Myom) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-Myosin antibody (red, 4A). Nuclei were stained with DAPI (blue, 4A). There were no organized sarcomeres in the *rybp*^{-/-} CMCs (4A) on day 21. The *myom-1* gene expression level was two times higher in the *rybp*^{-/-} cells (4B).

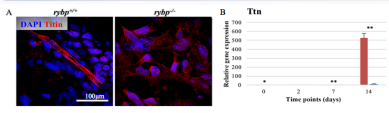
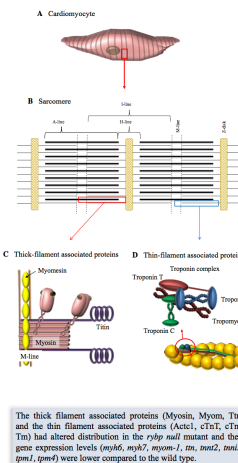


Figure 5: Titin ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-Titin antibody (red, 5A). Nuclei were stained with DAPI (blue, 5A). The *rybp*^{-/-} CMCs did not have any organized sarcomeres (5A) on day 21. The *ttn* gene expression level was fifty times higher in the *rybp*^{-/-} cells (5B).

THE SARCOMERE

The CMCs are rod-shaped striated muscle cells with one or two nuclei.

The red square represents a sarcomere unit (2A). The sarcomere is the smallest functional unit of the striated 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 2B, pink 2C) and thin filaments (actin) (thin line 2C, yellow 2D) and associated proteins. The red square shows a half-sarcomeric unit with thick-filament associated proteins and the blue square shows a half-sarcomeric unit with thin-filament associated proteins (2D).



The thick filament associated proteins (Myosin, Myom, Ttn) and the thin filament associated proteins (Actin, cTnT, cTnI, TnI) had altered distribution in the *rybp* null mutant and their gene expression levels (*myh6*, *myh7*, *myom-1*, *ttn*, *tnnl2*, *tnnl3*, *tpst1*, *tpst2*) were lower compared to the wild type.

Thin filament associated proteins (D)

The thin filaments are consisted of two actin (alpha cardiac actin) (mustard yellow) strands, which contains actin-myosin binding sites [7]. Nebulin (violet green) is a molecular template for the actin formation and specifies the actin filament length [8]. Tropomyosin (dark red) wraps around the actin, it has essential role in the Ca²⁺-mediated contraction [9], because it contains the Ca²⁺ binding troponin complex, including cardiac Tropomyosin I (blue), C (green) and T (red) [10]. Alpha-actinin (α-actinin) (aquamarine) anchors actin and ctn are located in the Z-disc, crosslinks the neighbouring sarcomeres [11].

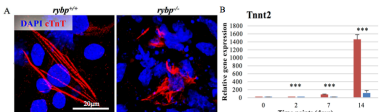


Figure 6: Cardiac Tropomyosin I (cTnI) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-cardiac Tropomyosin I antibody (red, 6A) showing shorter and there were no sarcomeric structure in the *rybp*^{-/-} CMCs (6A) it was clearly visible in the *rybp*^{+/+} cultures on day 21. Nuclei were stained with DAPI (blue, 6A). qRT-PCR analysis showed that the *ttni2* gene was significantly downregulated in the *rybp*^{-/-} cells (6B).

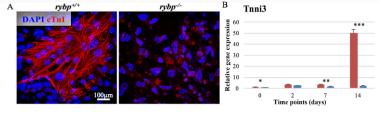


Figure 7: Cardiac Tropomyosin I (cTnI) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-cardiac Tropomyosin I antibody (red, 7A). Nuclei were stained with DAPI (blue, 7A). Dense, filamentous, striated structures were visible in the *rybp*^{+/+} CMCs while there were less protein in the cytoplasm of the *rybp*^{-/-} CMCs (7A) on day 21. The *ttni3* gene was downregulated in the lack of Rybp (7B).

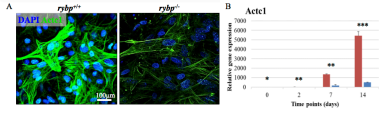


Figure 8: Alpha cardiac actin (Actin) ICC and qRT-PCR analysis of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells. CMCs were stained with anti-alpha cardiac actin I antibody (red, 8A). Nuclei were stained with DAPI (blue, 8A). Dense, filamentous, well-organized structures were visible in the *rybp*^{+/+} CMCs while there were less protein in the lack of Rybp (8B).

What might be the role of Rybp during cardiac differentiation?

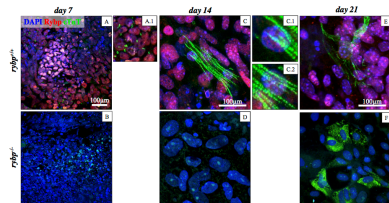


Figure 9: Rybp and Cardiac Tropomyosin I (cTnI) ICC of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells on day 7, 14 and 21.

CMCs were stained with anti-Rybp antibody (red, 9A) and anti-cTnI antibody (green, 9B). Nuclei were stained with DAPI (blue, 9A). Rybp and cTnI were colocalized in the nuclei of differentiating *rybp*^{+/+} cells (white, 9B). In most cases, the Rybp showed different intensities in the nuclei (9A,C,D) and the cTnI protein was out of the nucleus in small dots (9A,A). There were no detectable Rybp signal in the *rybp*^{-/-} cells (9C,D) and the amount of cTnI protein was lower than in the *rybp*^{+/+} cells (9D). The amount of Rybp protein was lower in the nuclei of differentiated CMCs than in the undifferentiated cells on day 14 and day 21 (9C,E,F). The cTnI protein did not show organized sarcomeric pattern on day 14 and day 21 in the *rybp*^{-/-} cells (9D,F).

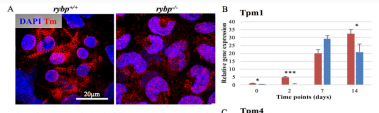


Figure 10: Rybp and Cardiac Tropomyosin I (cTnI) ICC of CMCs derived from *rybp*^{+/+} and *rybp*^{-/-} ES cells on day 7, 14 and 21.

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SUMMARY

Sarcomerogenesis is impaired in the absence of Rybp during *in vitro* cardiac differentiation. Rybp is important for the (1) normal expression, (2) subcellular localization, (3) assembly of sarcomeric components (4) and for subsequent sarcomerogenesis (1/). (5) 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 therapeutic applications.

Figure 11: The hypothesized role of Rybp in the sarcomerogenesis during cardiac differentiation

