Polycomb protein RYBP regulates transcription factor *Plagl1* during *in vitro* cardiac differentiation of mouse embryonic stem cells

Ph.D. Thesis Booklet

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INTRODUCTION

Mouse RING1 and YY1 binding protein (RYBP) is a core member of the non-canonical polycomb repressive complexes 1 (ncPRC1s), which are classically highlighted for their role as repressors. RYBP is also described as a protein with the ability to interact to multiple partners playing roles in diverse functions. Our laboratory has demonstrated the essential role of RYBP in early mouse embryonic development. We have previously also reported that mouse embryonic stem (ES) cells lacking RYBP could not form functionally contracting cardiomyocytes (CMCs) *in vitro* and that Pleiomorphic adenoma gene-like 1 (*Plagl1*) was one of the most downregulated genes in the *Rybp*$^{-/-}$ ES cells and differentiated CMCs as identified by genome wide transcriptome analysis.

Plagl1 is a cardiac transcription factor shown to be expressed in a chamber restricted manner in the developing mouse embryonic heart. *Plagl1* is also determined to co-express with key cardiac transcription factors Nk2 homeobox 5 (*Nkx2-5*) and Myocyte enhancer factor 2c (*Mef2c*) in the developing mouse embryonic heart. Impaired expression of *Plagl1* is implicated to the formation of congenital heart disorders (CHD).

During my PhD work, I have further characterized the role of *Plagl1* during CMC development *in vitro* and demonstrated that *Plagl1* is a downstream target of RYBP. I determined that in wild type conditions *Plagl1* was first expressed from the progenitor stage and expressed prominently during the CMC formation stages of *in vitro* cardiac differentiation. I identified that RYBP activates the

expression of *Plagl1* by associating with cardiac transcription factor NKX2-5 during the process of cardiomyogenesis. My results also demonstrate that the two non-coding RNAs (ncRNAs) *Hymai* and *Plagl1it* participates in the regulation of *Plagl1* promoters.

AIMS OF THE STUDY:

The main goal of this thesis study was to characterize the functions of RYBP during *in vitro* cardiac differentiation with focus on understanding the regulatory activities RYBP exerts at the *Plagl1* locus.

The detailed aims of the thesis were:

i. To examine the expression of *Plagl1* and to compare it to the expression of *Rybp* during *in vitro* cardiac differentiation.

ii. To characterize and compare the protein localisation of RYBP and PLAGL1.

iii. To identify putative regulatory elements in the *Plagl1* genomic locus.

iv. To define the nature of the regulatory mechanism which RYBP exerts on the *Plagl1* locus.

METHODS

In vitro cardiac differentiation of mouse ES cells:

Mouse wild type (*Rybp*$^{+/+}$) and *Rybp null* mutant (*Rybp*$^{-/-}$) ES cells were cultured on gelatin coated tissue culture dishes. Cardiac differentiation of ES cells was performed by the hanging drop method as described in Keller, 1995. The cultured embryoid bodies by the hanging drop method were collected on day 2, plated on gelatin coated dishes and cultured for a maximum of 21 days. Samples were derived for further analyses at different days (d) of cardiac differentiation: d0, 2, 7, 10, 14 and 21.

Molecular analysis for expression kinetics:

We performed relative gene expression analysis and studied protein localisation in the pluripotent ES cells and differentiating CMCs at the designated time points of *in vitro* cardiac differentiation. Relative gene expression analysis was performed for *Plagl1*, *Hymai* and *Plagl1it* by quantitative real-time PCR (qRT-PCR). Protein levels of PLAGL1 was studied by Western blot analysis. To study the protein abundancy and spatiotemporal distribution of RYBP, PLAGL1 and sarcomere protein cardiac Troponin T (CTNT) in the differentiated cardiac cultures, immunocytochemistry (ICC) was performed.

Chromatin Immunoprecipitation:

To check the ability of RYBP to bind at the *Plagl1* gene promoters, chromatin immunoprecipitation (ChIP) was performed and the binding levels were measured by qRT-PCR.

Molecular cloning:

Plagl1 P1 and *P2* promoters were amplified from BAC construct RP23-259L24 and cloned into pGL4.20 vector. *Tnnt2* promoter was amplified from genomic DNA isolated from wild type ES cells and cloned into pGL4.20 vector. Expression constructs for *Hymai*, *Plagl1it*, NKX2-5 and MEF2C were amplified from whole cell cDNA pool generated from wild type d10 CMCs and were cloned into pcDNA3.1 – and pRK7FLAG vectors respectively.

Co-Immunoprecipitation:

Protein interaction studies were performed by transiently transfecting HEK293T cells with overexpression constructs of RYBP and NKX2-5 and co-immunoprecipitation was performed with antibodies against RYBP and FLAG. The immunoprecipitated samples were run on Western blot to confirm interaction.

Protein stability assays:

Stability of PLAGL1 protein was performed by treating the wild type d14 differentiated cardiac cultures with either 75 µg/ml Cycloheximide or 10 µM MG132. The cell lysates were procured on

an hourly basis until 6 hours (hrs) i.e., 1 hr, 2 hr, 3 hr, 4 hr, 5 hr and 6 hr and the samples were analysed by Western blot.

Luciferase reporter assay:

HEK293T cells were transfected with the calcium phosphate transient transfection method to study *Plagl1* and *Tnnt2* promoter activities in the absence or presence of regulatory transcription factors (RYBP, *Hymai*, *Plagl1it*, NKX2-5, MEF2C and PLAGL1). The transfected cells were harvested for protein cell lysates 40 hours after transfection. Samples were analysed for luciferase activity of the transfected promoter constructs with Perkin Elmer TopCount NXT Luminometer in dark conditions.

Bioinformatic analysis:

Genome wide transcriptome analysis was performed by hierarchical gene clustering using K-means clustering method and the heatmaps of the generated gene clusters were performed in PRISM Graphpad 8. Analysis of the reported ESTs of the *Plagl1* splice variants was performed by downloading all available *Plagl1* mRNA sequences in FASTA format and aligning them with the *Plagl1* genome using BioEdit. Transcription factor binding site analysis in the *Plagl1* promoters were achieved by using TRANSFAC and motif search in sarcomere gene promoter regions for PLAGL1, NKX2-5, MEF2C and TBX5 binding was performed in JASPAR. Metadata analysis in ES cells and differentiated CMCs for RYBP and RNF2 binding was achieved by using IGV_2.9.4.

SUMMARY OF FINDINGS:

Calcium homeostasis, the JAK-STAT pathway and cell adhesion are amongst the most affected pathways in the *Rybp*$^{-/-}$ ES cells and derived CMCs in comparison to the wild type cells

To get insights into the molecular mechanisms behind the non-contractility phenotype in the *Rybp* null mutant ES cells and its derived CMCs we performed genome wide transcriptomics using both wild type and *Rybp* null mutant cells during cardiac differentiation. Hierarchical gene clustering and functional enrichment analysis revealed that genes relating to ion channel, calcium ion homeostasis, the JAK stat pathway and cell adhesion as the most affected mechanisms in the *Rybp* null mutant during the time course of *in vitro* cardiac differentiation.

Cardiac progenitor formation and sarcomere organisation are impaired in the *Rybp* mutant CMCs

Further analysis of the transcriptome for genes that play crucial roles in cardiac development revealed that genes required for the formation of cardiac progenitor and sarcomere organisation expressed at reduced levels in the *Rybp* mutant CMCs.

Plagl1 is expressed from its _P1_ and _P3_ promoters during CMC formation

Plagl1 has a complex genomic structure with three promoters, eleven exons and two non-coding RNAs *Hymai* and *Plagl1it* located in the intronic regions of the locus. Our EST analysis identified that all three promoters in the *Plagl1* genomic locus can produce protein coding transcripts. By using primers specific to the various splice variants corresponding to the promoter regions, we determined that *Plagl1* is expressed only from its *P1* and *P3* promoters in the wild type cells. As expected *Plagl1* was not expressed either in the *Rybp* null mutant ES cells or CMCs during *in vitro* cardiac differentiation.

In the wild type cells, *Plagl1* was expressed first from d4 which is the early cardiac progenitor formation stage and expressed highly at d14 suggesting its important role in CMC formation.

Interestingly, the ncRNAs exhibited a severely impaired expression pattern as *Plagl1* itself, suggesting that the two ncRNAs may function during cardiac differentiation and might play role in the regulation of *Plagl1* itself.

RYBP activates _Plagl1_ via its _P1_ and _P3_ promoters in a polycomb independent mechanism

By performing luciferase reporter assays, we determined that RYBP activates *Plagl1 P1 and P3* promoters. By treating the cells with PRT4165 a PRC1 inhibitor, we also identified that RYBP activated the promoters in a polycomb independent mechanism. Analysis of

existing ChIP-seq data for the binding of RYBP and its polycomb cofactor RNF2 in chromatin collected from ES cells and cardiac progenitor cells revealed the RNF2 independent binding of RYBP at the *P3* promoter in the cardiac progenitor cells and not in ES cells.

RYBP activates the *Plagl1 P3* promoter by interacting with cardiac transcription factor NKX2-5

To identify the molecular mechanism by which RYBP activated the *Plagl1 P3* promoter, I subcloned the *P3* promoter into various deletion mutants. In order to check the inducibility of the *P3* deletion mutants by RYBP I performed luciferase reporter assays and determined that the 3' of the *P3* promoter was activated the highest by RYBP. Transcription factor binding site (TFBS) analysis revealed that the 3' of the *P3* promoter contained consensus binding sites for cardiac transcription factors NKX2-5 and MEF2C. By performing site directed mutagenesis at the identified *Nkx2-5* and *Mef2c* sites we confirmed that the *Nkx2-5* sites were critical for the activation of the *P3* promoter by RYBP. The mutation of *Mef2c* site did not attenuate the activation levels of the *P3* promoter by RYBP suggesting that this site was not important for the activation of the promoter by RYBP.

RYBP interacts with NKX2-5 to activate *Plagl1 P3* promoter

To examine if RYBP interacted with NKX2-5 to activate the *P3* promoter, co-immunoprecipitation was performed. The results

confirmed that RYBP interacted with NKX2-5 to activate the *P3* promoter.

ChIP-qPCR using primers specific to the *Nkx2-5* and *Mef2c* consensus sites in ES cells and d7 CMCs revealed that RYBP can bind at the *Nkx2-5* sites in both the *P1* and *P3* promoter in d7 CMCs and not in ES cells.

***Hymai* and *Plagl1it* ncRNAs synergistically affect the activation of the *Plagl1 P3* promoter by NKX2-5**

To determine if *Hymai* and *Plagl1it* can synergistically affect *P3* activation by NKX2-5, luciferase reporter assay was performed by co-transfecting HEK293T cells with *P3* promoter in combination with *Hymai* or *Plagl1it* and NKX2-5. Our results determined that both *Hymai* and *Plagl1it* significantly enhanced the activation of *P3* promoter by NKX2-5.

PLAGL1 co-expressed and activated *Tnnt2* expression via its promoter

To determine if the loss of *Plagl1* expression contributed to the non-contractility phenotype in the *Rybp*$^{-/-}$ mutant CMCs, ICC analysis of d7 and d14 wild type cardiac cultures were performed by staining the samples with PLAGL1 and cardiomyocyte marker and sarcomere thin filament protein CTNT. The analysis showed that PLAGL1 and CTNT co-expressed in the differentiating CMCs indicating the potential role of PLAGL1 in the formation of terminally differentiated cardiomyocytes.

To analyse if PLAGL1 can transcriptionally affect *Tnnt2* expression, luciferase reporter assay was performed by co-transfecting HEK293T cells with the *Tnnt2* promoter construct in combination with PLAGL1, NKX2-5 or MEF2C. Our results determined that besides NKX2-5, PLAGL1 can also activate the *Tnnt2* promoter. These results suggested that PLAGL1 might be also important for the formation of terminally differentiated cardiomyocytes, especially by regulating sarcomere gene expression.

CONCLUSIONS

In this thesis project I have investigated the molecular underpinnings of how RYBP affected *Plagl1* expression during *in vitro* cardiac differentiation.

By utilising ES cells based *in vitro* cardiac differentiation system and comparing the wild type and the *Rybp* null mutant cells differentiation abilities for gene expression, protein production, protein localisation, I determined that in the lack of RYBP the expression of *Plagl1* and the ncRNAs *Hymai* and *Plagl1it* were severely reduced in the CMCs. This demonstrated that the entire *Plagl1* locus was affected by the loss of RYBP and suggested that RYBP might be important for the regulation of this locus. With the consequent experiments I have also shown that RYBP is able to activate the *Plagl1* locus via its *P1* and *P3* promoters. Finally, my experiments also demonstrated the RYBP associates with cardiac transcription factor NKX2-5 to achieve its regulatory functions.

My results also highlighted the important role *Plagl1* exerts in the formation of sarcomere organisation by regulating *Tnnt2*, revealing that the absence of *Plagl1* in the *Rybp*$^{-/-}$ CMCs may itself contribute to the non-contractility phenotype of this cell line. Since *Plagl1* first expressed from the progenitor formation stage of *in vitro* cardiac differentiation, future efforts will be required to identify its functions during this stage.

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LIST OF PUBLICATIONS

The thesis is based on the following publications

1) **Surya Henry**, Viktória Szabó, Enikő Sutus, Melinda K. Pirity., RYBP is important for cardiac progenitor cell development and sarcomere formation. *Plos One (2020)*
   doi: /10.1371/journal.pone.0235922

(Q1, I.F: 3.24)

2) Izabella Bajusz, **Surya Henry**, Enikő Sutus, Gergő Kovács, Melinda K. Pirity., Evolving role of RING1 and YY1 binding protein in germ cell specific transcription regulation. *MDPI Genes* (2019)
   doi: /10.3390/genes10110941

(Q1, I.F: 3.75)

Cumulative impact factor: 6.99

MTMT identifier: 10053037

DECLARATION

I declare that the contribution of Surya Henry was significant in the listed publications and the doctoral process is based on the publications listed. The results reported in the PhD dissertation and the publications have not been used to acquire any PhD degree previously and will not be used in the future either.

Szeged,

31.08.2021

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Dr. Melinda K. Pirity