Ph.D. THESIS

Gaining insights into the *in vivo* function of RYBP/DEDAF, a recently discovered polycomb protein: utilization of the mouse - as a genetically tractable mammalian model organism

Melinda Katalin Pirity

Supervisior: Dr. Attila Gácser

Ph.D. School of Biology

Department of Microbiology

Faculty of Science and Informatics

University of Szeged

Szeged

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Introduction

Present work summarizes findings on the *in vivo* function of the mouse *rybp*/Rybp (Ring1 and YY1-Binding Protein; also known as DEDAF, YEAF1; UniGene Mm.321633; MGI:1929059) gene/protein. Several *in vitro* functions (transcriptional regulator, mediator of apoptosis) have been implied from RYBP's protein interaction profile that includes DNA binding proteins (YY1), polycomb group proteins (Ring1A, Ring1B, M33 and mPC2), and members of the pro-apoptotic caspase pathways but the precise cellular and *in vivo* role of RYBP currently was not well understood.

Rybp is an evolutionarily conserved protein with a zinc-finger motive that was identified first as an interacting partner for the Polycomb group protein Ring1A (Ring1; ortholog of Drosophila dRing/Sce) and also was shown to associate with Ring1B (Ring2/Rnf2; ortholog of Drosophila dRing/Sce) and M33 (Pc1; ortholog of Drosophila Pc). Polycomb group (PcG) proteins function as transcriptional repressors acting part through histone modification, and are believed to be important regulators of organogenesis and cell lineage specification. Rybp engages PcG proteins, transcriptional corepressors that participate in the establishment of a stably silenced state of key developmental (e.g. hox) and proliferation genes. These three PcG members function as components of the PRC1 multiprotein complex that maintains the repressed state of loci that have previously been “marked” for repression via the histone methyltransferase activity of the PRC2 PcG initiation complex.

Because Rybp can bind to PRC1 proteins as well as to several sequence-specific transcription factors, it has been classified as an adapter protein that can recruit the PcG proteins to these factors and the specific genetic loci they regulate. As an aside, it has also been proposed that Rybp can serve as a bridging factor between two DNA binding transcription factors, although it should be noted that one of the partners is often YY1, which itself has been classified as a PcG component. An Rybp-related protein, Yaf2 (55% identity on the amino acid level), also has been shown to engage PRC1 components and DNA binding transcription factors.

Although the precise molecular function of Rybp is not yet known, these interactions suggest that Rybp may be a multifunctional developmental regulator. To investigate the biological role of the multifunctional Rybp protein, we have analyzed Rybp expression patterns in developing mouse embryos and targeted Rybp for deletion in the mouse using homologous recombination in embryonic stem (ES) cells. Furthermore, this thesis describes complementary strategies of *(1)* targeted mutagenesis and *(2)* conditional ectopic transgenic alleles to achieve loss- and gain-of-function, respectively in order to reveal *in vivo* functions of Rybp in the developing mouse. As a result of these studies, exciting phenotypes have emerged in these models, and present ongoing and future efforts to pursue them further.

Aims

A major focus of my work was to ask the following questions and to complete the next studies:

• Is Rybp essential for mouse embryonic development?

• If not, can homologue Yaf2 substitute its function? Are the biological functions of Rybp and Yaf2 redundant?

• If yes, Rybs is essential for mouse embryonic development what is the exact function?

• What is the phenotype of the RYBP loss-of-function mouse?

• What is the phenotype of the RYBP gain-of-function mouse?

• Characterization of the RYBP loss-of-function mouse

• Characterization of the RYBP gain-of-function mouse

• Do the phenotypes correspond to the expression pattern of Rybp during mouse embryonic development? Where Rybps expressed ?

• What is the way of Rybp's action ?

Methods

Construction of the *rybp* targeting vectors

Rybp conditional transgenics

Generation and characterization of heterozygous null ES cell lines.

Generation of *rybp* mutant mouse lines and genotyping

Production of *rybp*$^{-/-}$ ES cells.

ES cell maintenance

ES cell electroporation

Histology and immunohistochemistry

Electron microscopy

Quantitative RT-PCR

Southern blots

Western blots

Cell death assay

Blastocyst outgrowth assay

Chimera production

Es cell injections, mouse breeding, husbandry and genotyping

Results

- The expression pattern of Rybp during mouse development was analysed and mice bearing a null mutation by gene targeting in embryonic stem cells was generated.
- Rybp was broadly expressed during embryogenesis, being particularly abundant in extraembryonic tissues including trophoblast giant cells.
- Consistent with this, *rybp* homozygous null animals exhibited embryonic lethality at peri-implantation stages suggesting that Rybp was essential for survival of the embryo, for the establishment of functional extraembryonic structures, and for the execution of full decidualization.

• Analysis of intercross matings between heterozygous null mice revealed an under-representation of recovered heterozygous null offspring. This semi-penetrant phenotype in the Rybp heterozygous null mice was observed in four independently targeted lines.

• Functions for Rybp in the brain were further supported by the finding of exencephaly in about 15% of *rybp* heterozygous mutant embryos, and by Rybp's distinct neural expression pattern.

• The presence of Rybp-deficient cells in the developing central nervous system resulted in forebrain overgrowth and in localized regions of disrupted neural tube closure.

• With a second, complementary approach, a conditional ectopic allele for Cre-mediated misexpression of Rybp was generated. To do this the non-essential and ubiquitous *ROSA26* locus was targeted with a floxed neo cassette followed by a functional RYBP-EGFP fusion cDNA. In this mice a silent mutation was introduced where the Rosa26 locus was targeted homologous manner in ES cells.

• The mutation was introduced to the mouse germ line and the silent mutation could be induced by crossing these mice with cre reporter lines.

• Applying this strategy two different mice were generated: one expressing Rybp ubiquitously and the other expressing it tissue specific manner (under the control of AlphaA-Crystallyn promoter) in the mouse lenses.

- The strategy was also designed that after induction all mice expressed Rybp in a fusion with enhanced green fluorescent protein (EGFP) allowing easy monitoring of the expression pattern.
- The results demonstrated that loss of a single *rybp* allele in conventional knockout mice often resulted in retinal coloboma, an incomplete closure of the optic fissure, characterized by perturbed localization of Pax6 but not of Pax2.
- In addition, about one half of Rybp-/- <-> Rybp+/+ chimeric embryos also developed retinal colobomas and malformed lenses.
- Tissue-specific transgenic overexpression of Rybp in the lens resulted in abnormal fiber cell differentiation and severe lens opacification with increased levels of Ap-2α and Sox2, and reduced levels of βA4-crystallin gene expression.
- Ubiquitous transgenic overexpression of Rybp in the entire eye caused abnormal retinal folds, corneal neovascularization, and lens opacification. Additional changes included defects in anterior eye development.
- These studies have shown that aberration in the normal protein levels of Rybp can result in retinal coloboma. Therefore, these studies established *rybp* as a novel gene that has been associated with coloboma, a developmental disease of the eye.
- We propose that the multiple functions for Rybp in regulating mouse retinal and lens development are mediated by genetic, epigenetic and physical interactions between these genes and proteins.

Conclusion

Together these data supported critical roles for Rybp at multiple stages of mouse embryogenesis and differentiation. Furthermore, however there is a possible overlap between Rybp and Yaf2 expression, obviously was not sufficient to prevent aberrant neurulation/neurogenesis and eye development resulting from reduced Rybp levels in our affected heterozygous and chimeric animals. These findings are likely to place Rybp in processes of differentiation, especially CNS and eye development, apoptosis and have implications for the understanding of neural tube defects, and neurodegenerative diseases. The cells and mice we have generated may allow us to assess the potential relationships between Rybp and these proteins and to place Rybp within known genetic networks. These tools also will provide opportunities for the elucidation of the precise molecular roles of Rybp as they relate to transcriptional regulation, apoptosis, and/or yet-to-be-identified cellular processes. In the future, the generation of conditional or tissue-specific knockout mice, with the CNS being an attractive focus, will allow us to understand more about Rybp's biological roles during development and in the context of aging and disease.

Publications

Peer-reviewed papers served as the basics for current thesis. :

1. He, S., **Pirity ,MK**., Wang, WL., Wolf, L., Chauhan, BK., Cveklova, K., Tamm, ER., Ashery- Padan, R., Metzger, D., Nakai, A., Chambon, P., Zavadil, J., and Cvekl, A. (2010) Chromatin remodeling enzyme Brg1 is required for mouse lens fiber cell terminal differentiation and its denucleation. *Epigenetics Chromatin.* Nov 30;3(1):21 (IF:4.67)

2. **Pirity, M.K**., WeiLin, W., Wolf, L., Tamm, E.R., Schreiber-Agus, N., Cvekl, A. (2007) Rybp, a polycomb group interacting protein required for mouse ocular development. *BMC Dev Biol. 30;7;39* (IF: 3.29)

3. **Pirity, M**., Locker, J., Schreiber-Agus, N. (2005) Rybp/DEDAF is required for early post- implantation and for central nervous system development. *Mol. Cell. Biol.* 16:7193-7202 (IF:6.057)

4. Dugast-Darzacq, C., **Pirity, M**., Blanck, J.K., Scherl, A., Schreiber-Agus, N. (2004) Mxi1- SRalpha: a novel Mxi1 isoform with enhanced transcriptional repression potential. *Oncogene* 23:8887-8899 (IF:7.135)

5. Nagy, A., Moens, C., Ivanyi, E., Pawling, J., Gertsenstein, M., Hadjantonakis, A-K., **Pirity, M**., Rossant, J. (1998) Multipurpose gene alterations from a single targeting vector: dissecting the role of *N-myc* in development. *Current Biol*. 8: 661-664 (IF:10.992)

Peer-reviewed papers not directly related to current thesis. :

6. **Pirity, MK.**, Dinnyes, A. (2010) Tbx3: another important piece fitted into the pluripotent stem cell puzzle. *Stem Cell Research & Therapy* 1:12.

7. Carstea, AC., **Pirity, MK.,** and Dinnyes, A. (2009) Germline competence of Mouse ES and iPS cell lines: chimera technologie and genetic background. *World Journal of Stem Cells* 1(1):22-29 (IF: new journal impact factor will be known in 2011)

8. Rungarunlert, S., Techakumphu, M., **Pirity, MK.,** and Dinnyes, A. (2009) Embryoid body formation from embryonic and induced stem cells: benefits of bioreactor. *World Journal of Stem Cells* 1(1):11-21. (IF: new journal impact factor will be known in 2011)

9. Kobolak, J., Kiss, K., Polgar, Z., Mamo, S., Roger-Gaillard, C., Tancos, Z., Bock, I., Baji, AG., Tar, K., Pirity, MK., Dinnyes, A. (2009) Promoter analysis of the rabbit *POU5F1* gene and its

expression in preimplantation stage embryos. *BMC Mol Biol.* Sep. 4;10:88 (IF:2.85)

10. *Rhee, J.M., ***Pirity, M.K**., *Lackan,' C.S., Long,' J.Z., Kondoh, G., Takeda' J., Hadjantonakis, A.-K. (2006) In vivo imaging and differential localization of lipid-modified GFP-variant fluorescent fusion proteins in embryonic stem cells and mice. *Genesis* 44:202-218 (IF:2.223)

11. Cole, M.J., **Pirity, M**., Hadjantonakis, A-K. (2003) Shedding light on Bioscience. *EMBO Rep.* 4:838-843 (IF:6.97)

12. Hever-Szabo, A., **Pirity, M**., Szathmari, M., Venetianer, A. (1998) P-glycoprotein is overexpressed and functional in severely heat-shocked hepatoma cells. *Anticancer Res.* 18: 3045-3048 (IF:1.65)

13. **Pirity, M**., Hever-Szabo, A., Venetianer, A. (1996) Overexpression of P-glycoprotein in heat- and/or drug-resistant hepatoma variants. *Cytotechnology* 19: 207-214 (IF:1.297)

14. Venetianer, A., **Pirity, M**., Hever-Szabo, A. (1994) The function of heat-shock proteins in stress tolerance. *Cell Biol. Int.* 18: 605-615 (IF:1.9)

15. **Pirity, M**., Nguyen, V.T., Dubois, M.F., Bensaude, O., Hever-Szabo, A., Venetianer, A. (1991) Decreased stress inducibility of the hsp68 protein in a rat hepatoma variant clone. *Eur. J. Biochem.* 210: 793-800 (IF:3.042)

Book chapters:

16. Hadjantonakis AK, **Pirity M**., Nagy A. (2008) Cre recombinase mediated alterations of the mouse genome using embryonic stem cells. *Methods Mol Biol*. 461:111-32.

17. **Pirity, M**., Blanck, J.K., Schreiber-Agus, N. (2006) Lessons learned from Myc-Max-Mad knockout mice. *Curr. Topics Microbiol. Immunol.* 302:205-234 (IF: 4.16)

18. Hadjantonakis, A-K., **Pirity, M**., Nagy, A. (1999) Cre recombinase mediated changes of the mouse genome using ES cells. Molecular Embryology: Methods and Protocols**.** *Methods in Molecular Biology* P.T. Sharpe and I. Mason Eds. 97: 101-138

19. **Pirity**, **M**., Hadjantonakis, A-K., Nagy, A. (1998) Embryonic stem cells: creating transgenic animals. Animal Cell Culture Methods. *Methods in Cell Biology.* J.P. Mather and D. Barnes Eds. 57: 279-293 (IF: 3.5)

***** Joint first co-authorship