

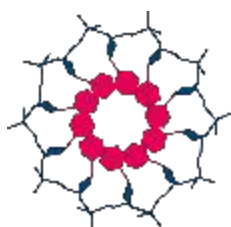
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P34

The SH3 domain of Caskin1 binds to lysophosphatidic acid suggesting a direct role for the lipid in intracellular signaling

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„ Src homology 3” or SH3 domains consist one of the most common protein domains in signal transduction, generally characterized by their binding to prolin-rich sequences on other signaling proteins [1, 2]. Caskin1, a scaffold protein regulating cortical actin filaments and enriched in neural synapses in mammals [3], has an atypical SH3 domain, the prolin-rich interacting partner protein of it could not be identified yet. Based on previous reports that several SH3 domains are able to bind phospholipids [4], we sought for lipid interacting partners of the SH3 domain of human Caskin1. We investigated the signaling-born lysophospholipid mediators sphingosine-1-phosphate and lysophosphatidic acid [5, 6], well-known first messengers activating G protein-coupled receptors [7, 8], but with mostly unknown intracellular protein targets, as potential binding partners for this SH3 domain. Here, we provide evidence that the SH3 domain of human Caskin1 selectively binds to lysophosphatidic acid in a biphasic manner in vitro. The binding strength and stoichiometry depend on the association-state of the lipid, as well as on the molecular subspecies determined by its fatty acid moiety. This in vitro observed interaction allows enhanced binding of Caskin1's SH3 domain to membranes if lysophosphatidic acid accumulates locally due to elevated production in signaling, fulfilling a second messenger-like role for lysophosphatidic acid. Our results suggest that the SH3 domain of human Caskin1 is a lipid binding domain rather than a prolin-rich motif interacting domain.

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P35

Internal motion of the PDZ1-PDZ2 tandem of PSD-95 represented by dynamic structural ensembles

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The PSD-95 (post synaptic density protein 95) is an intracellular protein found in the postsynaptic part of neurons. It plays an important role in organizing signal transduction processes. As a major scaffold protein, it anchors membrane receptor proteins in the postsynaptic membrane. Its first two PDZ domains, near the N-terminus, form a structurally and functionally independent subunit often referred to as a PDZ tandem. The PDZ1-PDZ2 tandem of PSD-95 has been proved to be rigid in its apo state; however, ligand binding to both domains induces elevated interdomain mobility [1]. The present study aims at the elucidation of the change in the internal motion of PDZ tandem upon ligand binding by means of dynamic structural ensembles. Structural ensembles are generated by restrained molecular dynamic simulation, where experimental NMR parameters [2], such as NOEs and Lipari-Szabo model-free order parameters (S^2) are imposed on the protein models. Detailed analysis of the ensembles is expected to shed light on the structural and dynamical rearrangements at the domain level leading to changes in interdomain mobility.

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P36

Rybp is required for the terminal differentiation of neural lineages

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Polycomb Group (PcG) family member Ring1 and Yy1 Binding Protein (Rybp) regulates diverse biological functions through both Polycomb-dependent and -independent mechanisms. We have previously reported that lack of Rybp causes early embryonic

lethality in mice and that a portion of heterozygotes does not have cerebellum, exhibit exencephaly and disorganized neurocortex. These studies demonstrated the essential *in vivo* role of Rybp in the development of the central nervous system (CNS). In this work, we used wild type (*rybp*^{+/+}) and *rybp* null mutant (*rybp*^{-/-}) mouse embryonic stem (ES) cells and differentiated them towards neural lineages *in vitro* with the purpose to uncover underlying molecular events that are responsible for the *in vivo* phenotypic changes. By using histological, gene expression and protein localization analyses we revealed that: *rybp*^{-/-} ES cells are able to differentiate to all three major germ layers and develop towards neural stem cells (NSCs) and neural precursors (NPCs), but they can not form matured neurons and glias. Furthermore, lack of *rybp* coincided with altered gene expression of key neural markers and transcription factors including Pax6 and Plagl1 pinpointing a possible transcriptional circuit among Rybp and these genes. Together, these findings support critical roles for Rybp in neural lineage commitment. Revealing the genetic underpinnings of differentiation has important implications not only for understanding neurological diseases, learning and memory but also for the possibility of neural repair through genetic reprogramming of non-neural cells to a neurogenic fate. This work was supported by TÁMOP-4.1.1.C-13/1/KONV-2014-0001 and TÁMOP-4.2.6.A-15/1-2015-0002.

P37

Investigation the protein family occurring most abundantly in the Drosophila sperm

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Drosophila sperm reaches an extraordinary longevity with 1.8 mm at the end of spermatogenesis. A mature sperm contains acrosome, elongated nuclei and the long tail part, which consists of axoneme and two elongated mitochondrial derivatives. Elongation of spermatids is driven by the extension of mitochondria and the microtubule array surround them. Organization of the mitochondrial derivatives and the function of them in the mature sperms are not fully known. Proteomic studies revealed that the Sperm-leucyl-aminopeptidase (S-Lap) protein family, with all eight members of it, is an abundant constituent of the mature sperm, however, their functions are unknown [1]. We have identified mutations in four S-Lap genes with male-sterile phenotype and characterized them genetically and morphologically. We used the CRISPR-Cas9 method to induce mutation in the remaining four S-Lap

genes. In spite of their similarity, there is no redundancy between the investigated S-Lap genes, and they should have important single role during spermatogenesis. We proved that the S-Lap proteins localized to the mitochondria and found that S-Lap mutants show mitochondrial structural abnormalities. We measured leucyl aminopeptidase activity and found that the lack of a single S-Lap protein does not affect the testis-specific enzyme activity, what confirms the assumption that the diverged members of the protein family gained new function during spermatogenesis. Funding: OTKA NF 101001, MTA Bolyai János Kutatási Ösztöndíj.

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P38

Inhibition profiling of retroviral protease inhibitors using an HIV-2 modular system

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Retroviral protease inhibitors (PIs) are fundamental pillars in the treatment of HIV infection and AIDS. Currently used PIs are designed against HIV-1, and their effect on HIV-2 is understudied. Using a modular HIV-2 protease cassette system, inhibition profiling assays were carried out for protease inhibitors both in enzymatic and cell culture assays. Moreover, the treatment-associated resistance mutations (I54M-L90M) were introduced into the modular system, and comparative inhibition assays were performed to determine their effect on the susceptibility of the protease. Our results indicate that darunavir, saquinavir, indinavir and lopinavir were very effective HIV-2 protease inhibitors, while tipranavir, nelfinavir and amprenavir showed a decreased efficacy. I54M-L90M double mutation resulted in a significant This is the first study to characterize in-depth the susceptibility of HIV-2 protease to all of the inhibitors, using a standardized protocol that allows for the comparative analysis of results obtained from enzymatic assays and those measured in cell culture

P39

A Serine protease inhibitor is involved in cellular response upon UV irradiation

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Rybp is required for the terminal differentiation of neural lineages

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Introduction

Neural differentiation of embryonic stem cells (Fig 1)
Ring1 and Yy1 Binding Protein (Rybp, UniGene Mm.321633; MGI:1929059) is a member of the non-canonical Polycomb Repressive Complex 1 (ncPRC1) which has important role during embryonic development. Alterations of Rybp dosage resulted in striking neural tube defects (NTDs) in mouse models (Pirity et al, 2005) suggesting its role in neural differentiation. Here, we investigated underlying molecular events of the previously described central nervous system (CNS) phenotype of the *rybp*^{-/-} mice and the role of Rybp in neural lineage specification using stem cell based *in vitro* model system.

Materials and methods

Neural differentiation of embryonic stem cells (Fig 1)
rybp^{+/+} (wild type) (Nagy et al, 1993) and *rybp*^{-/-} (null mutant) (Pirity et al, 2005) mouse embryonic stem (ES) cells were differentiated to neural lineages induced by retinoic acid (RA) (Sigma, Cat.No R2625-100MG) as described (Bibel et al, 2007). Samples were taken for gross morphology (Fig 2), qRT-PCR (Fig 3) and ICC (Fig 4) analysis on day 0 (d0), day 3 (d3), day 7 (d7), day 10 (d10) and day 14 (d14).

Quantitative real time PCR (qRT-PCR)

The isolated RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat.No 4368814). Primers were designed for the amplification of specific products (Gsc, Sox17, Fgf5, NeuroD1, NeuN, Olig2, Syn1, Psd95, Plagl1). qRT-PCR reactions in SYBR Green for CFX (Applied Biosystems, Cat.No 4472942) were carried out in Bioer LineGene Real-time PCR System (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. To calculate fold change, the values compared to undifferentiated samples (d0, *rybp*^{+/+}).

Immunocytochemistry (ICC)

The cells were fixed with 4% paraformaldehyde (Sigma, Cat.No P6148) and permeabilized with 0.2% TritonX-100 (Sigma, Cat.No T8787-250ML). Then sequentially incubated with the following primary antibodies: Nestin (Rat-401; Hybridoma Bank, Iowa, USA, 1:100), Pax6 (Pax6; Hybridoma Bank, Iowa, USA, 1:100), TuJ1 (Neuronal Class III β-Tubulin Polyclonal Antibody; Covance, Cat.No PR8-4359, 1:1000), Glap (Monoclonal Anti-Glial Fibrillary Acidic Protein Clone G-A-5, Sigma, Cat.No G3893, 1:400), Map2 (Anti-MAP2 antibody; Abcam, Cat.No ab52454, 1:1000), RC2 (RC2; Hybridoma Bank, Iowa, USA, 1:100), Tau (Tau (H-150), Santa Cruz Biotechnology, Cat.No sc-5387, 1:100) and 2H3 (2H3; Hybridoma Bank, Iowa, USA, 1:1000) and conjugated to fluorescently labeled secondary antibodies (Alexa Fluor 488 Goat-Anti-Mouse, Invitrogen, Cat.No A-10667; Alexa Fluor 647 Donkey-Anti-Mouse, Invitrogen, Cat.No A-31571; Alexa Fluor 647 Goat-Anti-Rabbit, Invitrogen, Cat.No A-21244) and counterstained with DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories, Cat.No H-1200).

Results

Results are shown on Fig 2 - Fig 4.

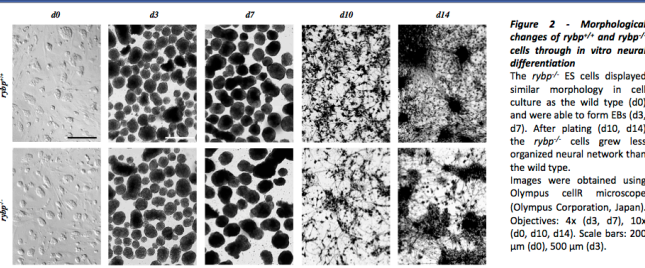


Figure 2 - Morphological changes of *rybp*^{+/+} and *rybp*^{-/-} cells through *in vitro* neural differentiation
The *rybp*^{-/-} ES cells displayed similar morphology in cell culture as the wild type (d0) and were able to form EBs (d3, d7). After plating (d10, d14) the *rybp*^{-/-} cells grew less organized neural network than the wild type. Images were obtained using Olympus cellR microscope (Olympus Corporation, Japan). Objectives: 4x (d3, d7), 10x (d0, d10, d14). Scale bars: 200 μm (d0, d10, d14), 500 μm (d3, d7).

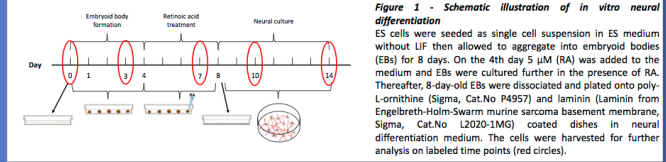


Figure 1 - Schematic illustration of *in vitro* neural differentiation
ES cells were seeded as single cell suspension in ES medium without LIF then allowed to aggregate into embryoid bodies (EBs) for 8 days. On the 4th day 5 μM (RA) was added to the medium and EBs were cultured further in the presence of RA. Thereafter, 8-day-old EBs were dissociated and plated onto poly-L-ornithine (Sigma, Cat.No P4957) and laminin (Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane, Sigma, Cat.No L2020-1MG) coated dishes in neural differentiation medium. The cells were harvested for further analysis on labeled time points (red circles).

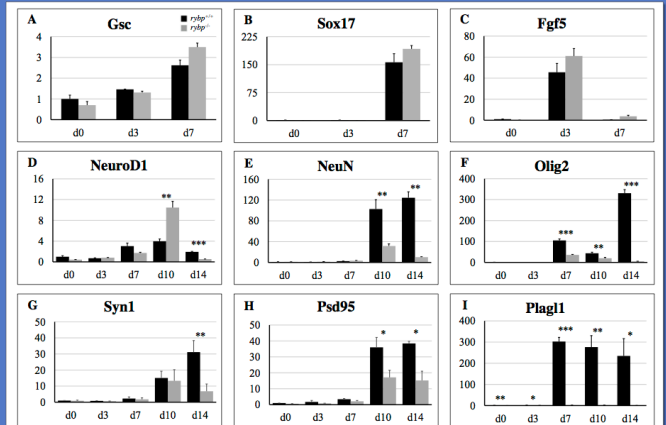


Figure 3 - Relative gene expression changes during *in vitro* neural differentiation
Relative gene expression analysis of germline markers Gsc (mesoderm) (A), Sox17 (endoderm) (B) and Fgf5 (primitive ectoderm) (C) showed no differences. Relative gene expression analysis of early neural marker NeuroD1 (D) by qRT-PCR showed elevated expression in *rybp* null mutant neural cultures in comparison to wild type, meanwhile late neural marker NeuN (E), oligodendrocyte marker Olig2 (F), presynaptic marker Syn1 (G) and postsynaptic marker Psd95 (H) downregulated in *rybp*^{-/-} neural cultures compared to wild type. qRT-PCR also showed that induction of neural transcription factor Plagl1 (I) was deficient in the *rybp*^{-/-} cells. The expression of the indicated markers was normalized to Hprt expression. Means are standard deviation ± SD. Values of p < 0.05 were accepted as significant (* p < 0.05; ** p < 0.01; *** p < 0.001). Statistical method: t test type 3.

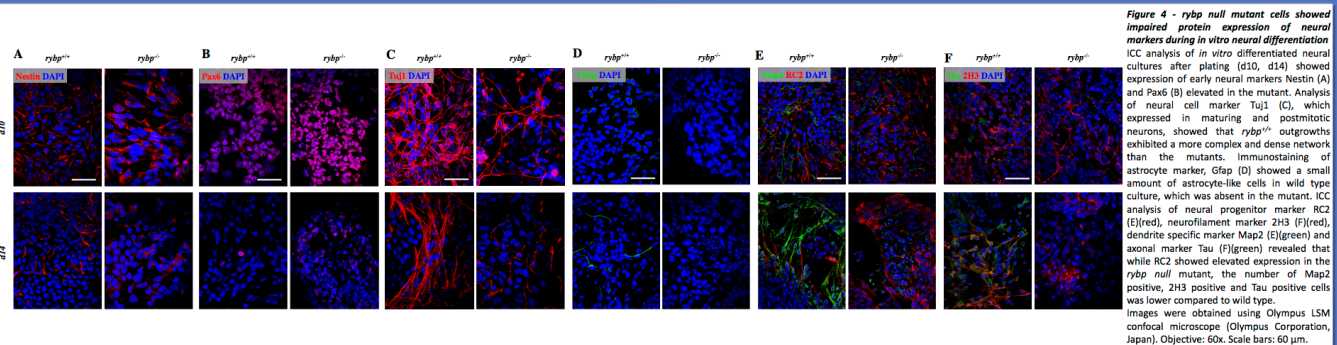


Figure 4 - *rybp* null mutant cells showed impaired protein expression of neural markers during *in vitro* neural differentiation
ICC analysis of *in vitro* differentiated neural cultures after plating (d10, d14) showed expression of early neural markers Nestin (A) and Pax6 (B) elevated in the mutant. Analysis of neural cell marker TuJ1 (C), which expressed in maturing and postmitotic neurons, showed that *rybp*^{-/-} outgrowths exhibited a more complex and dense network than the mutants. Immunostaining of astrocyte marker, Glap (D) showed a small amount of astrocyte-like cells in wild type culture, which was absent in the mutant. ICC analysis of neural progenitor marker RC2 (E)(red), neurofilament marker 2H3 (F)(red), dendrite specific marker Map2 (E)(green) and axonal marker Tau (F)(green) revealed that while RC2 showed elevated expression in the *rybp* null mutant, the number of Map2 positive, 2H3 positive and Tau positive cells was lower compared to wild type. Images were obtained using Olympus LSM confocal microscope (Olympus Corporation, Japan). Objective: 50x. Scale bars: 60 μm.

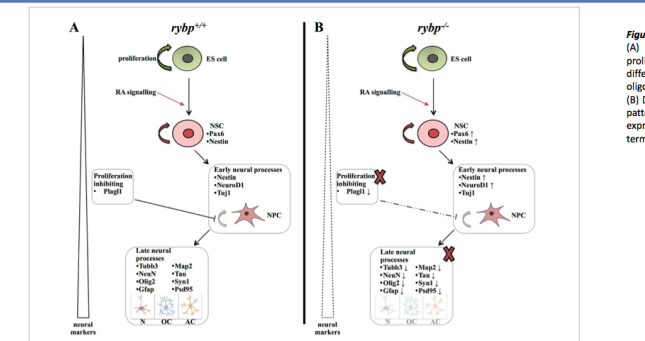


Figure 5 - Model of dosage dependent effect of Rybp during *in vitro* neural differentiation of ES cells
(A) Markers of early neural processes (Pax6, Nestin, NeuroD1) are upregulated in neural stem cells (NSCs), which will confer reduced proliferative and high differentiation capacity to the cells. In the presence of Rybp, upregulation of Plagl1 helps to facilitate late neural differentiation and inhibits cell proliferation, which will facilitate the generation of terminally differentiated neural cell types (N: neurons, OC: oligodendrocytes, AC: astrocytes).
(B) During neural differentiation of pluripotent ES cells in the absence of Rybp, markers of early neural processes exhibit elevated expression pattern. These will facilitate the generation of a bigger pool of neural precursors (NPCs) with higher proliferative capacity. In the mutant, Plagl1 expression is defective, which contributes to increased proliferative and decreased differentiation capacity of the NPCs. The formation of terminally differentiated neural cell types will be impaired in the lack of functional Rybp (Kovacs et al, 2015).

Summary

We showed here that normal level of Rybp is indispensable for the proper differentiation of neural cell types. Lack of Rybp accelerated NPC formation, whilst obscured terminal differentiation of neural lineages. The formation of neurite processes and their organization into complex network also influenced by the presence of Rybp. In the absence of functional Rybp it is very likely that the balance of early and late neural processes are changed. These results confirm our hypothesis that Rybp is essential for the development of mature neural cells. Together, these results support a continuing role for Rybp as regulator of neural circuit formation and maintenance, above and beyond its developmental function as regulator of neuronal and glial fate.

Acknowledgements

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