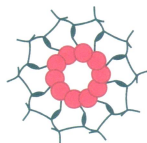
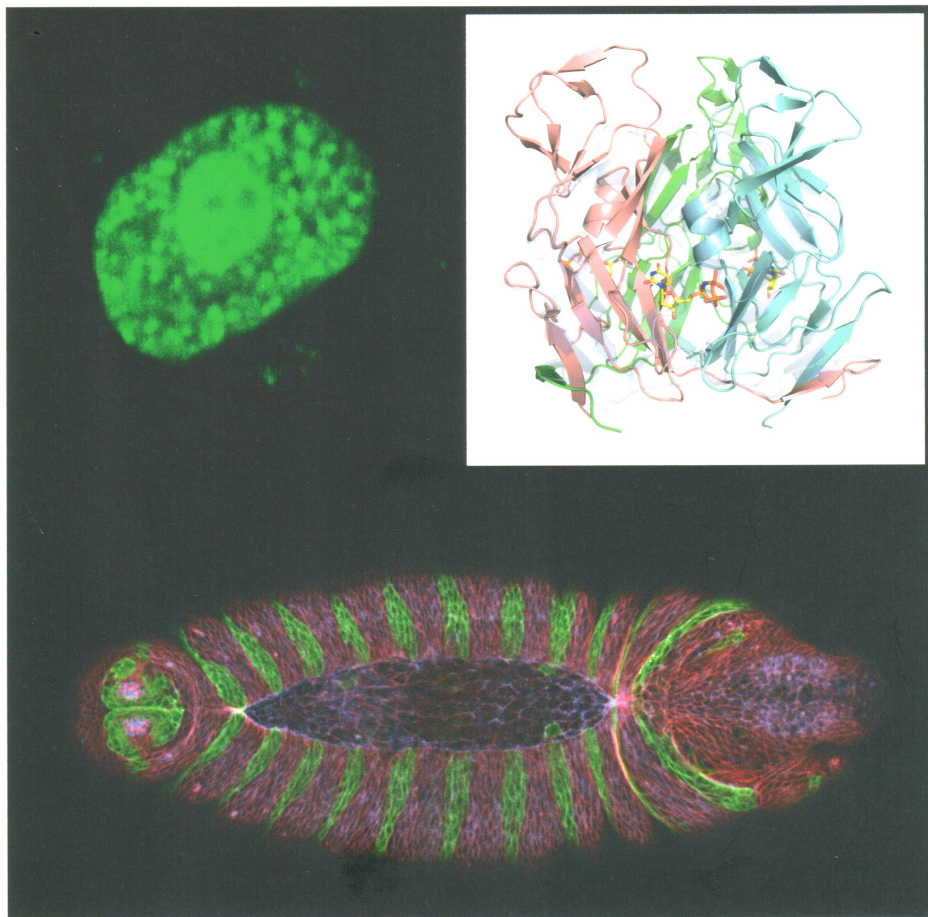


Hungarian Molecular Life Sciences 2015

27-29 March 2015



Hungarian
Biochemical
Society



**Programme &
Book of abstracts**

ISBN 978-615-5270-15-4



These data can explain the essential role of syndecan-4 in both skeletal muscle development and regeneration.

P-155

LACK OF RYBP IMPAIRS NEURAL DIFFERENTIATION OF EMBRYONIC STEM CELLS

Gergő Kovács, Viktória Szabó, Melinda K. Pirity

Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary

Rybp (Ring1A and Yy1 Binding Protein) is a member of the non-canonical Polycomb Repressive Complex 1 with essential role in early embryonic development. We have previously reported that alterations of Rybp dosage in mouse models induced striking neural tube defects (NTDs), exencephaly, disorganized neurocortex and coloboma of the eye. However, the exact molecular mechanism responsible for the *in vivo* phenotypes remained unknown.

We aimed to uncover underlying molecular events that are responsible for the observed phenotypic changes. We used mouse embryonic stem (ES) cells as *in vitro* models of development. Since *in vitro* neural differentiation processes follow the *in vivo* neuroectodermal development, we utilized wild type (*rybp*^{+/+}) and *rybp* null mutant (*rybp*^{-/-}) ES cells and differentiated them towards neural lineages *in vitro*. We used retinoic acid (RA) to facilitate neural development. Differentiation revealed clear morphological differences between the wild type and the *rybp* null mutants the latter consistently producing poor neuron-like outgrowths in cultures. Further molecular and immunostaining studies confirmed altered expression of key neural, glial markers (e.g. Pax6, Nestin, Tuj1, Olig2) and members of the RA signal transduction pathway (e.g. Stra6, Crabp2) suggesting that *rybp* has important effect on neural lineage entry by ES cells. In the *rybp* null mutants, we have also found a complete defect in the induction of tumor suppressor Plagl1/Lot1/Zac1, which is an important regulator of neural differentiation processes. Furthermore, Plagl1 was the first gene unrelated to tumor suppressor p53 that was found to regulate cell cycle progression and apoptosis, these two fundamental genetic programs. In the *rybp*^{-/-} cells the expression of several cell-cycle regulators (e.g. Ccnd2, p21) are also altered. Thus, our findings showed that normal dosage of Rybp is indispensable for the proper neural differentiation and lack of functional Rybp severely impairs the formation of neural cell types.

Keywords: *embryonic stem cells, neural differentiation, Rybp, Plagl1*

LACK OF RYBP IMPAIRS NEURAL DIFFERENTIATION OF EMBRYONIC STEM CELLS

Gergő Kovács, Viktória Szabó, Melinda K. Pirity

Biological Research Centre, Hungarian Academy of Sciences, Szeged



INTRODUCTION

Ring1 and Yy1 Binding Protein (Rybp, UniGene Mm.321633; MGI:1929059) is a member of the non-canonical Polycomb Repressive Complex 1 and the BCOR (BCL6 corepressor) complex. It has essential role in embryonic development. Alterations of Rybp dosage cause striking neural tube defects (NTDs) in mouse models (Pirity *et al.*, 2005). Here, we characterized the possible molecular mechanisms underlying this phenotype.

MATERIALS AND METHODS

Neuronal differentiation of embryonic stem cells (Fig 1)

Rybp^{+/+} (wild type) (Nagy *et al.*, 1993) and *rybp*^{-/-} (mutant) (Pirity *et al.*, 2005) mouse embryonic stem (ES) cells were differentiated to neural lineages induced by retinoic acid (RA) according to Bibel *et al.*, 2007.

Immunocytochemistry (ICC)

The cells fixed with 4% paraformaldehyde and permeabilized with 0.2% TritonX-100. Then sequentially incubated with the following primary antibodies: Nestin (Rat-401; Hybridoma Bank, Iowa, USA, 1:100), Tuj1 (Neuronal Class III β -Tubulin Polyclonal Antibody; Covance, Cat.No PRB-435P, 1:1000) and Zac1 (Zac1 (M-300); Santa Cruz Biotechnology, Cat.No sc-22812, 1:1000) and labeled with fluorescent-labeled secondary antibody (Alexa Fluor 647 G-A-R IgG, Invitrogen, Cat.No A-21244) and DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories, Cat.No H-1200).

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 (Cnd2, Crabp1, Crabp2, Gfap, Nanog, Nestin, NeuroD1, Oct4, Olig2, p21, Pax6, Rara, Stra6, Tubb3, Zac1). qRT-PCR reactions in SYBR Green for CFX (Applied Biosystems, Cat.No 4472942) were carried out in Bioer LineGeneK Real-time PCR System (Bioer, China). Relative gene expression changes were quantified using the $\Delta\Delta Ct$ 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*^{+/+}).

RESULTS AND CONCLUSIONS

Rybp^{+/+} and *rybp*^{-/-} ES cells were *in vitro* differentiated to neural lineages. Samples were taken for gross morphology (Fig 2A), ICC (Fig 2BCD) and qRT-PCR (Fig 3) analyses on day 0 (d0), day 3 (d3), day 7 (d7), day 10 (d10) and day 14 (d14).

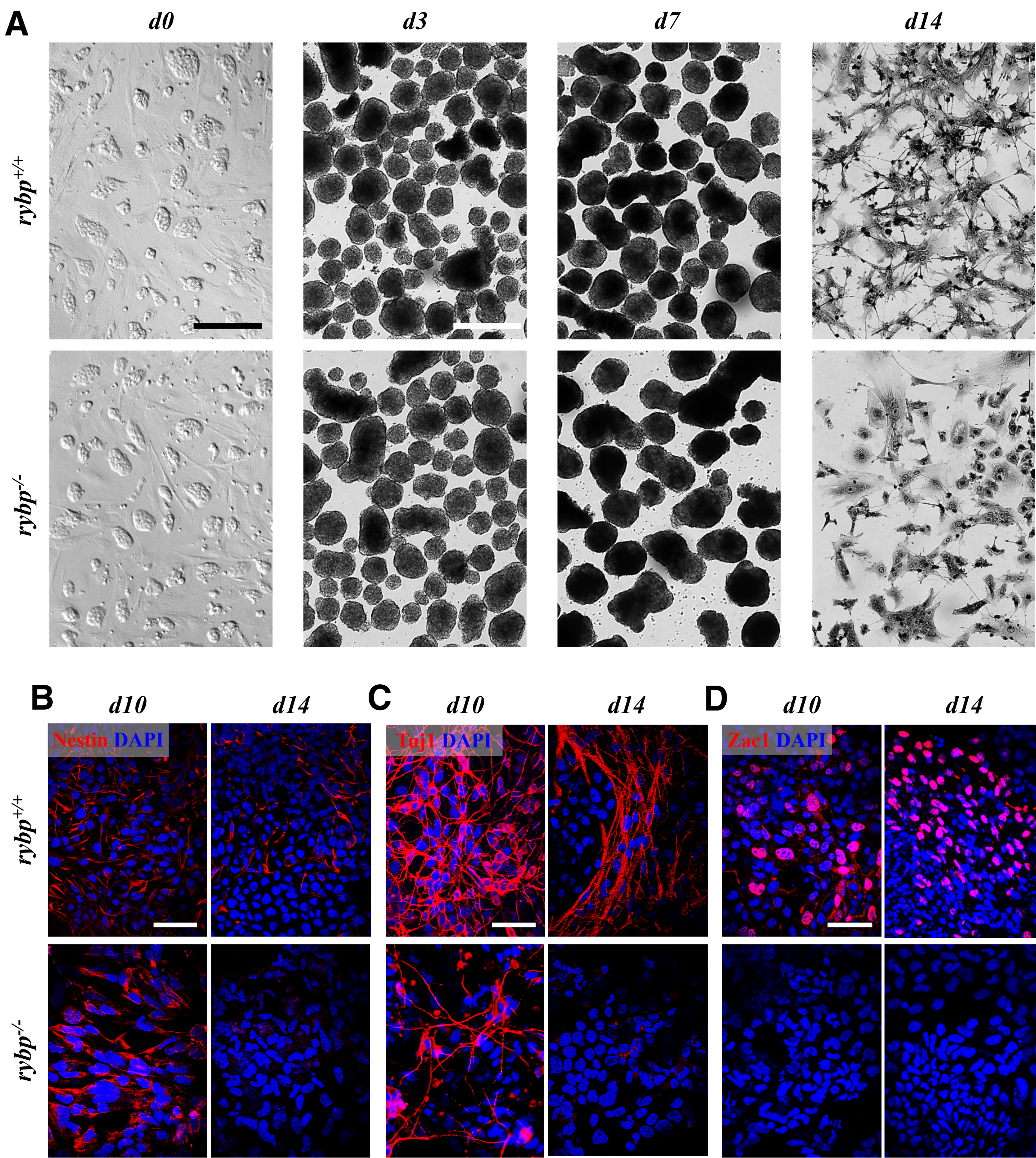


Figure 2 – EB formation and ICC analyses of the *rybp*^{+/+} and *rybp*^{-/-} ES cells during *in vitro* neural differentiation
(A) EB formation (d3, d7) is not compromised in the *rybp*^{-/-} cells but they grew less neurite processes (d10, d14) in comparison to the wild type. Images were obtained using Olympus cellR microscope (Olympus Corporation, Japan). Magnifications are 4x (d3, d7) and 10x (d0, d14). Scale bars represent: 200 μ m (d0) and 500 μ m (d3).
(B) Neural precursor (NPC) marker Nestin is abundantly presented in the *rybp*^{-/-} neurons (d10) suggesting an accelerated precursor production in the mutant cells. By d14 Nestin expression declined in both cell lines. (C) Decreased amount of neurite processes leads to the formation of a less complex networks in the *rybp*^{-/-} neural cultures revealed by Tuj1 immunostaining. (D) Zac1 immunostaining reveals lack of cell-cycle and apoptosis regulator Zac1 (Abdollahi *et al.*, 1997; Spengler *et al.*, 1997) in the *rybp*^{-/-} neural cultures. Images were obtained using Olympus LSM confocal microscope (Olympus Corporation, Japan). Magnification is 60x. Scale bars represent 60 μ m.

SUMMARY

Our results demonstrate that normal level of Rybp is indispensable for the proper differentiation of neural cell types. In the absence of functional Rybp it is very likely that the balance of early and late neural processes are changed, which is probably linked to the upregulation of Pax6 and the complete loss of Zac1. The formation of neurite processes and their organization into complex network also influenced by the presence of Rybp. These results confirm our hypothesis that Rybp plays important role in the development of mature neurons. 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

This work was supported by NKTH-OTKA FP7 “Mobility” HUMAN-MB08C-80205.

References

Abdollahi A, Roberts D, Godwin AK, Schultz DC, Sonoda G, Testa JR, Hamilton TC. 1997. *Identification of a zinc-finger gene at 6q25: a chromosomal region implicated in development of many solid tumors.* Oncogene 14:1973-1979.
Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M, Goetz M, Barde Y-A. 2004. *Differentiation of mouse embryonic stem cells into a defined neuronal lineage.* Nature neuroscience 7:1003-1009.
Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. 1993. *Derivation of completely cell culture-derived mice from early-passage embryonic stem cells.* Proceedings of the National Academy of Sciences of the United States of America 90:8424-8428.
Pirity MK, Locker J, Schreiber-Agus N. 2005. *Rybp/DEDAF is required for early postimplantation and for central nervous system development.* Molecular and cellular biology 25:7193-7202.
Spengler D, Villalba M, Hoffmann A, Pantaloni C, Houssami S, Bockaert J, Journot L. 1997. *Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain.* The EMBO journal 16:2814-2825.

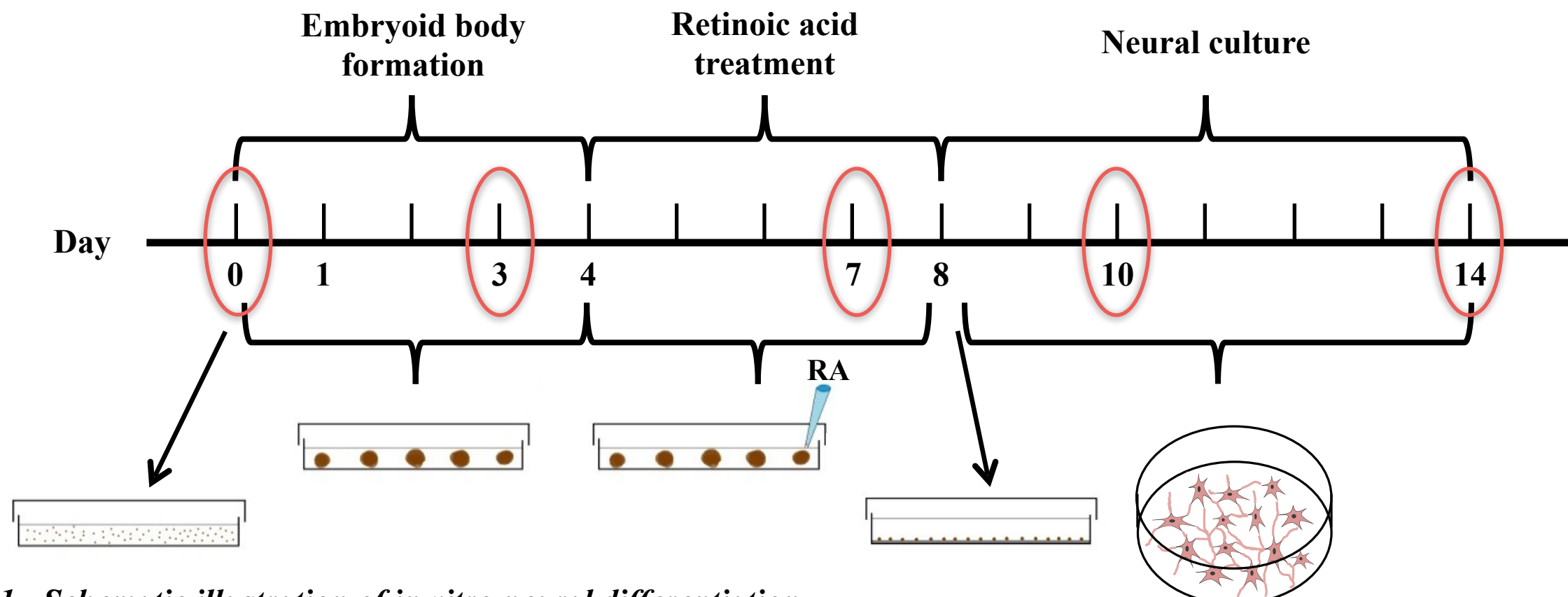


Figure 1 - Schematic illustration of *in vitro* neural differentiation
ES cells seeded as single cell suspension in ES medium without LIF then allowed to aggregate and form embryoid bodies (EBs) for 8 days. On the 4th day 5 μ M (RA) was added to the medium and EBs were cultured for a further 4 days in the presence of RA. Thereafter, 8-day-old EBs were dissociated and plated onto L-ornithine and laminin coated dishes in neural differentiation medium. The cells were harvested for further analyses on labeled time points (red circles).

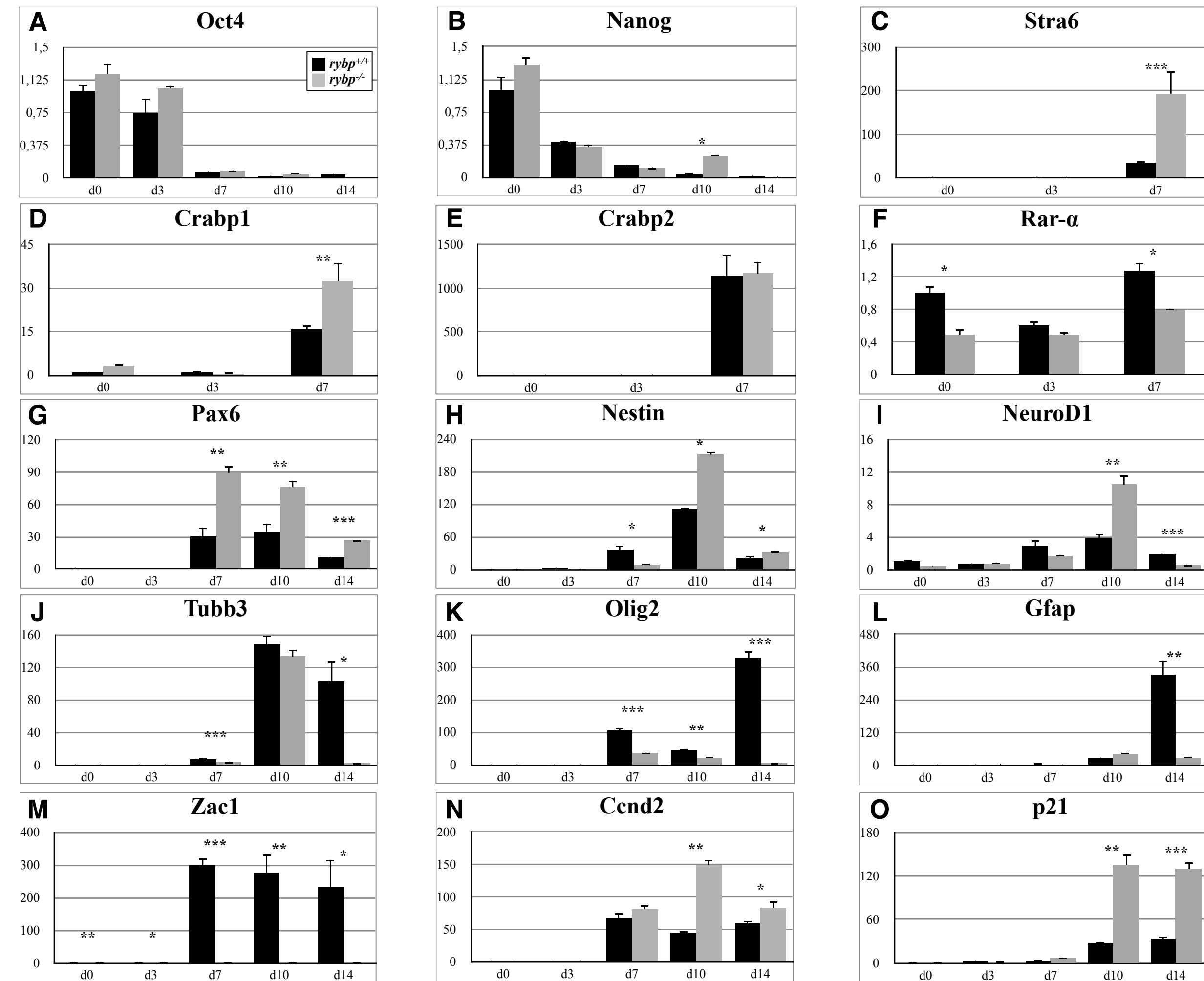


Figure 3 - Relative gene expression changes during *in vitro* neural differentiation
Oct4 (A) and Nanog (B) expression is downregulated in both cell lines suggesting proper silencing of pluripotency markers during differentiation. Crabp2 (E) and Rar α (F) expression is unchanged but Stra6 (C) and Crabp1 (D) expression is higher in *rybp*^{-/-} cells compared to the wild type suggesting an increased RA sensitivity of the *rybp*^{-/-} cells. The expression of early neural markers (Pax6 (G), Nestin (H), NeuroD1 (I)) is elevated after neuroectodermal induction by RA (d7, d10) then decreased by the end of differentiation (d14). The expression of early neural markers was higher generally in the *rybp*^{-/-} cells. On the contrary, the expression of late neural markers (Tubb3 (J), Olig2 (K), Gfap (L)) are deficient (d14) in the mutant. These data suggest that the mutant cells form a larger NPC pool than wild-type cells (d10) but the formation of mature neural cell types (d14) from this pool is defective. Zac1 (M) is strongly induced after the RA treatment (d7, d10, d14) in the wild type but in the mutant its level remained low. The induction of cell-cycle markers (Cnd2 (N), p21 (O)) reaches higher peaks in the *rybp*^{-/-} cells (d7). These changes may cause altered cell-cycle processes in the mutant during *in vitro* neural differentiation (see Figure 4). Means are standard deviation $\pm$ 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.

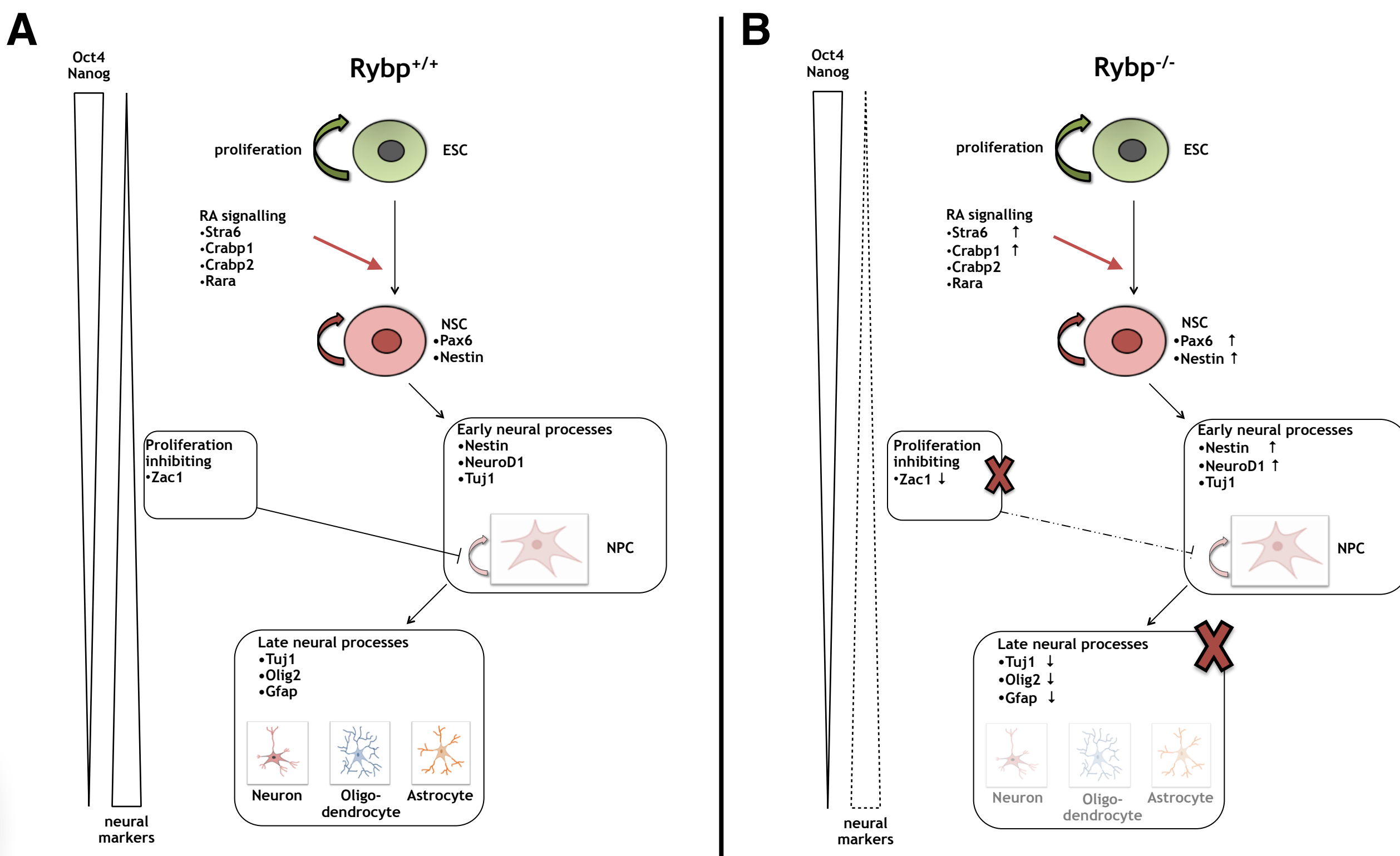


Figure 4 - Model of dosage dependent effect of Rybp during *in vitro* neural differentiation of ES cells
(A) Pluripotent ES cells express key pluripotency markers (e.g. Oct4, Nanog) in undifferentiated state, which are gradually decreasing during differentiation. Early neural markers (Pax6, Nestin) are 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 Zac1 helps to facilitate late neural differentiation and inhibits cell proliferation, which will facilitate the generation of terminally differentiated neural cell types.
(B) During neural differentiation of pluripotent ES cells in the absence of Rybp, RA pathway members and early neural markers exhibit elevated expression pattern. These will facilitate the generation of a bigger pool of NPCs with higher proliferative capacity. In the mutant, Zac1 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.