

Dosage dependent effect of Rybp on the retinoic acid mediated signalling pathway

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Background

Neurulation, subsequent growth and patterning of the neural tube are immensely complex processes (Fig.1). The neural tube forms as a single layer of pseudostatified epithelium which then proliferates rapidly and differentiation of neurons takes place laterally in the mantle layer. Patterning of the growing neural tube occurs in both the rostrocaudal axis and the dorsoventral axis under the influence of extracellular morphogens such as fibroblast growth factors (Fgfs), retinoic acid (RA), Sonic hedgehog (Shh) and bone morphogenetic proteins (Bmps) (Jessell, 2000).

Retinoic acid (RA) is the oxidized form of retinol (vitamin A), which is essential for embryogenesis and development of central nervous system (CNS). RA first appears in the mouse embryo at the mid-primitive streak to the late allantoic bud stage (E7.5) and it is an important inductive signal for neuronal differentiation (Fig.2). It is crucial for patterning the neural plate and the CNS.

Ring1A and YY1 Binding Protein (Rybp, UniGene Mm.321633; MGI:1929059) is an evolutionary conserved polycomb protein with essential role in embryonic development. Previous results showed that reduced Rybp level in mice (*rybp*^{-/-}) cause neurulation defects manifesting of neural tube defects which are the second most common congenital anomaly in humans (Pirty, 2005).

RA has been widely used as a potent inducer of *in vitro* neural differentiation in cell culture systems. Here we investigated the role of RA during *in vitro* neural differentiation by examining embryoid body (EB) formation and gene expression of wild type and *rybp* deficient mouse embryonic stem cells (ESCs) after RA induction.

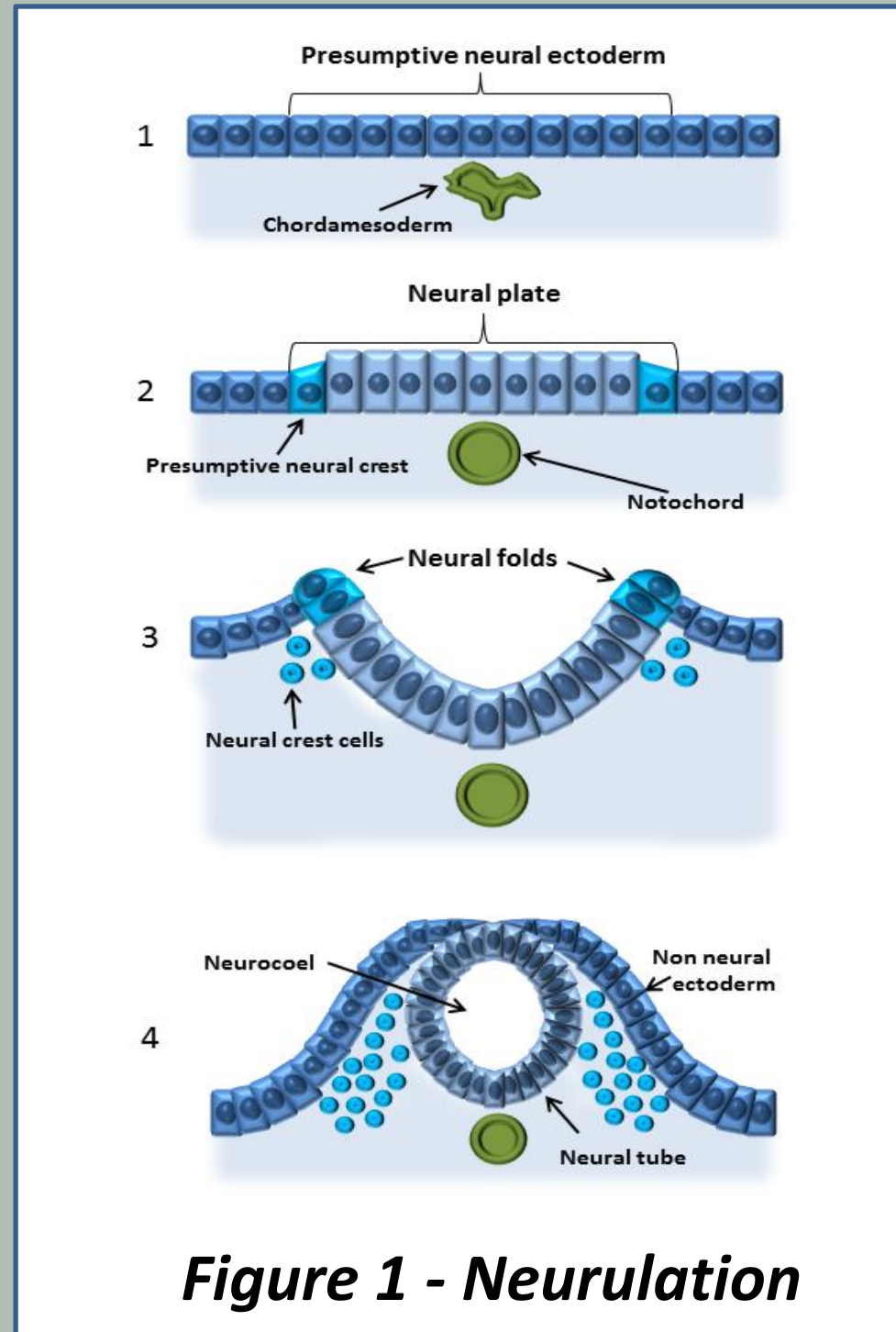


Figure 1 - Neurulation

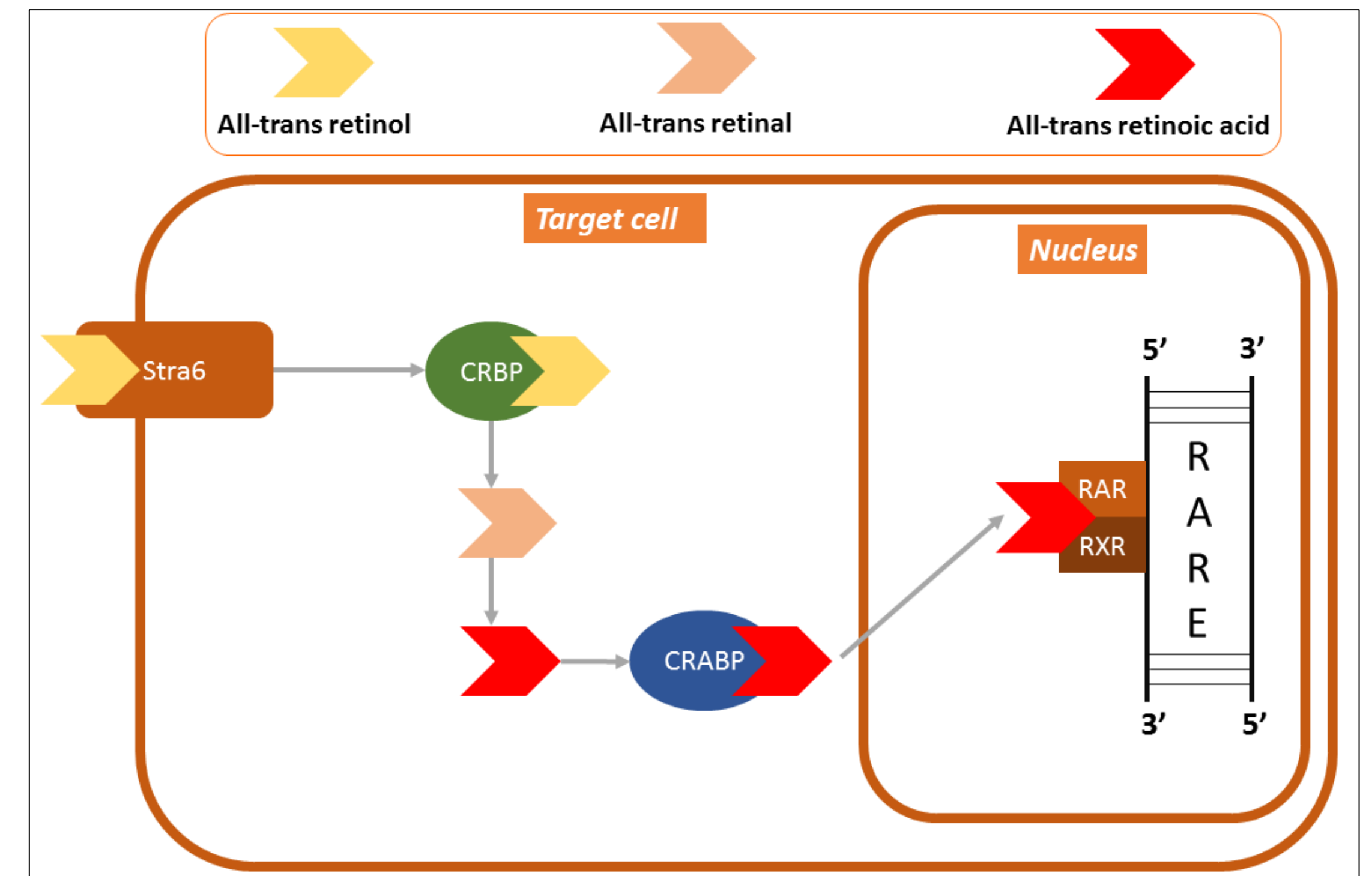


Figure 2 – Schematic presentation of the RA pathway

Stra6 (Stimulated by retinoic acid 6) is a transmembrane receptor which is responsible for the cellular uptake of retinol. The intracellular retinol binds to a complex formed by cellular retinol binding proteins (Crbp) after that the retinol converted to retinal than RA. The protein complex formed cellular RA binding proteins (Crabp) only binds intracellular RA with high affinity.

Rarα (Retinoic acid receptor, alpha) is part of the nuclear RA receptor complex member which regulates transcription in a ligand-dependent manner. Rarα binds as a heterodimer with Rxr (Retinoid X receptor) to the RA response elements (RARE) in their target genes in the absence of ligand and prevents transcription. Upon RA binding, Rar/Rxr release RARE and the target gene transcription initiated.

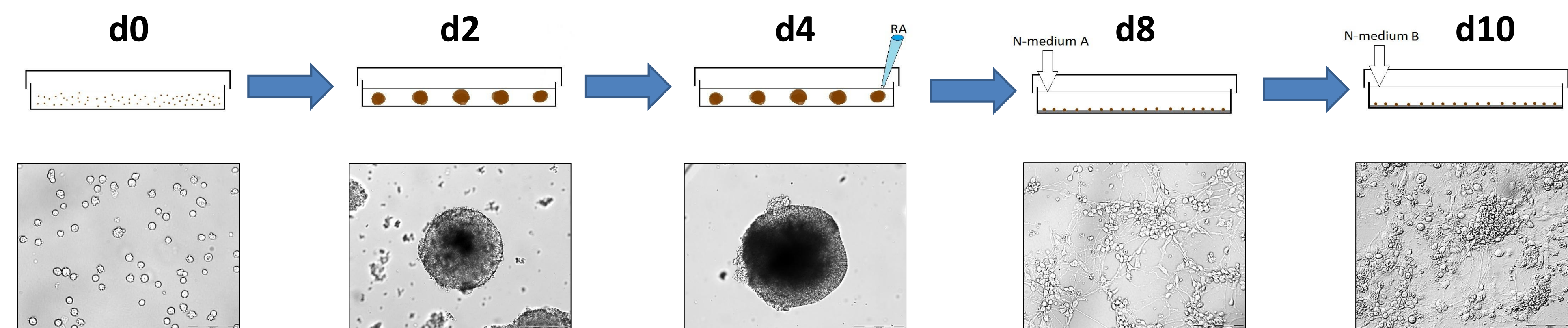


Figure 3 – Differentiation of mouse ES cells to neural lineages

ES cells were dissociated with 0.05% trypsin-EDTA solution to form a single cell suspension (day 0; d0) for Embryoid Body (EB) formation (day 2; d2). The EB is a common intermediate during the *in vitro* differentiation of pluripotent stem cells into specialized cell types. EBs treated with retinoic acid (RA) (day 4; d4), then dissociated with trypsin solution and plated as single cell suspension to new dishes (day 8; d8). The media was changed to neuronal differentiation medium A (N medium A) than N-medium B on day 10 (d10) (Bibel, 2007).

Methods

Neuronal differentiation of ES cells

Rybp^{+/-} (R1, wild type, control) (Nagy, 1993), *rybp*^{-/-} (D11, homozygous mutant) (Pirty, 2005) and *Tg^{rybp}* (C8, *rybp* overexpressing) (Pirty, 2007) mouse ESCs were differentiated to neuronal lineage according to Bibel, 2007. (Fig.3) EBs were sampled on day 3 (d3) and day 7 (d7) for embedding and sectioning (Fig.5). Sampling for qRT-PCR was performed on day 0 (d0), day 3 (d3) and day 7 (d7) (Fig.6).

EB sectioning

Harvested EBs were washed with phosphate-buffered saline and fixed with 4% (v/v) paraformaldehyde (PFA) for 15 min at room temperature on day 3 and 7. The PFA fixed EBs are embedded with HistoGel (Thermo Scientific, HG-4000-012). The solidified HistoGel was placed in tissue processor cassette, dehydrated with acetone, cleared it in xylene, infiltrated it with molten paraffin. Cell blocks were cut on Thermo Scientific HM325 Rotary Microtome at 3-4 µm. The sections mounted on microscope slides, stained with hematoxylin-eosin (H&E) (Fig.5A) and stained with Tuj1 (anti-Tubb3, Covance, PRB-435P, 1:5000) in order to reveal the formation of neurite processes (Fig.5B). The sections were scanned with Panoramic DESK system. On the scanned sections the diameter of the EBs were measured and the average sizes were calculated (Fig.4) from all genotypes (*rybp*^{+/-}, *rybp*^{-/-}, *Tg^{rybp}*) at both time points (d3, d7).

Real time PCR (qRT-PCR)

RNA was isolated from the samples using Qiagen RNeasy Mini Plus Kit according to the manufacturer's protocol. The RNA was reverse-transcribed using high capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) to produce cDNA. Primers were designed for the amplification of specific products (Crabp1, Crabp2, Stra6, Rarα). qRT-PCR reactions in SYBR Green for CFX (Applied Biosystems, 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*^{+/-}).

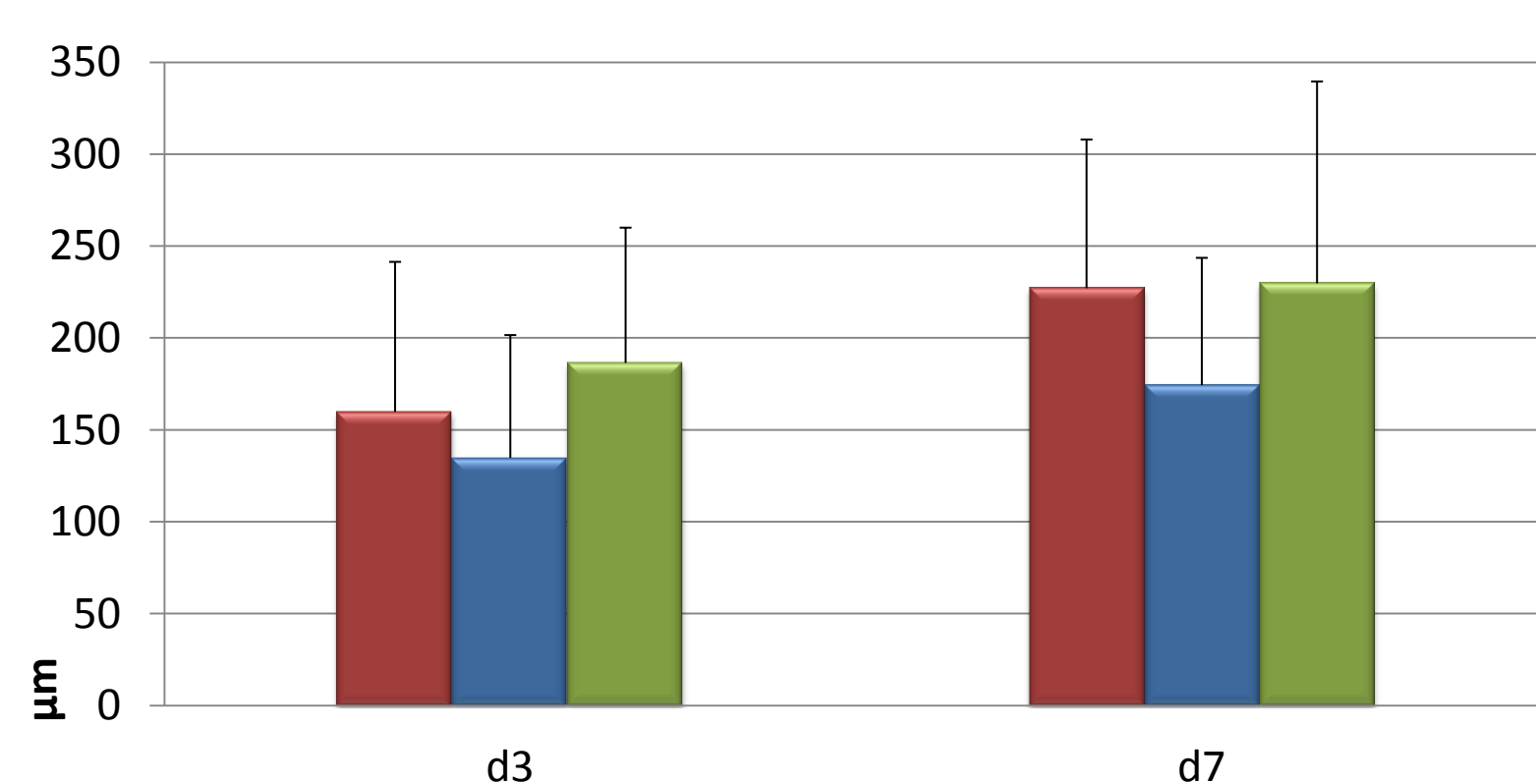


Figure 4 – Average size of EBs

EBs derived from ES cells showed various sizes (70-350 µm). EBs derived from *rybp*^{-/-} cells were smaller and EBs derived from *Tg^{rybp}* cells were larger than the *rybp*^{+/-} EBs. As expected, by d7 the average size of EBs were larger than at d3 at all three genotypes. Error bars represent standard deviation.

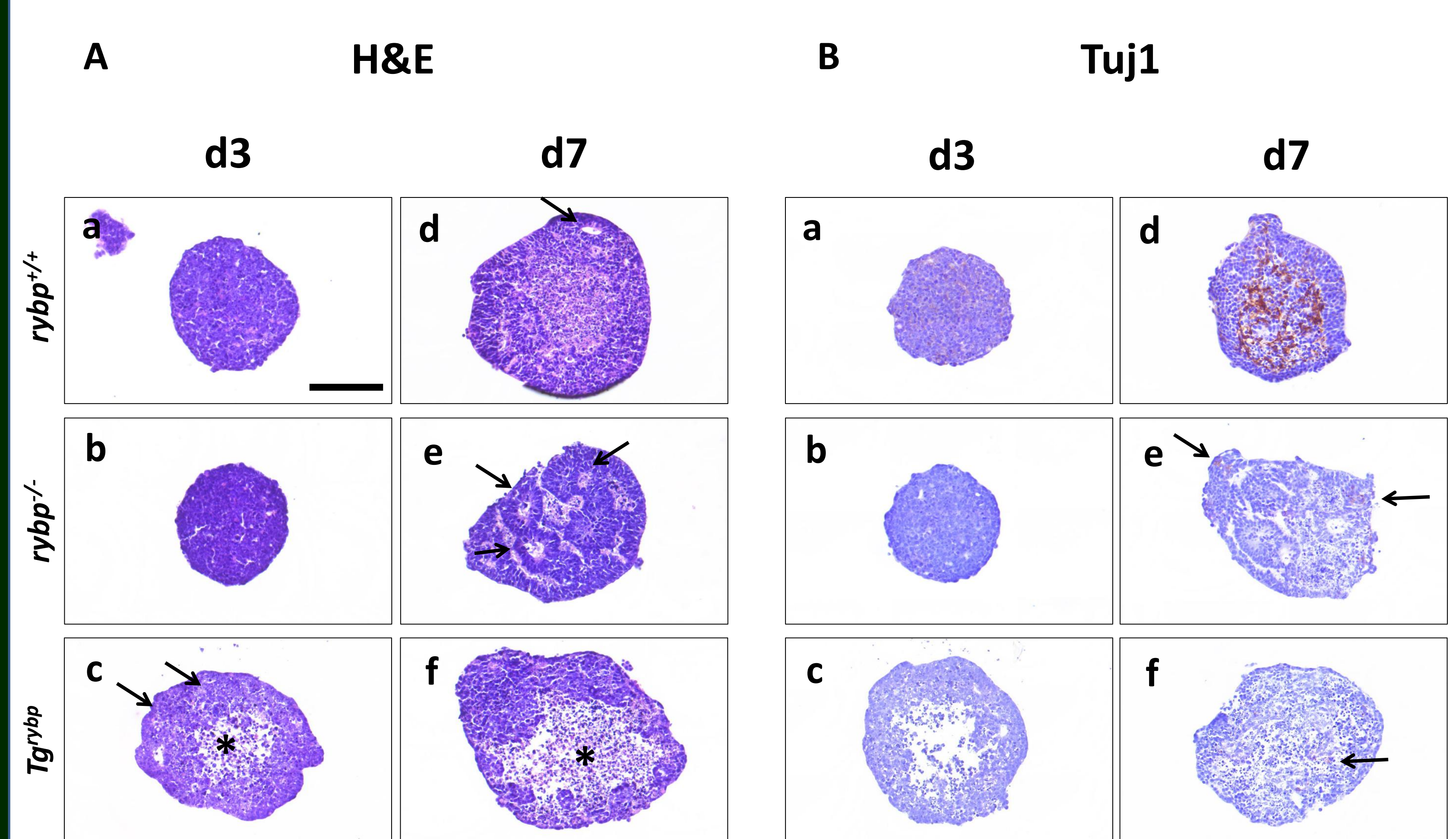


Figure 5 – Stem cell neural differentiation impairment in *rybp* mutant EBs

(A) H&E staining (purple) shows accelerated neuroepithelium formation in the *rybp*^{-/-} EBs (A – e; black arrows) compared to EBs derived from *rybp*^{+/-} ES cells (A – d, black arrow). The formation of ectoderm and neuroepithelium is impaired in the *Tg^{rybp}* EBs (A – c, black arrows), hence the process of cavitation (black stars) was accelerated in *Tg^{rybp}* EBs by both examined timepoints (A – c, f). (B) Tuj1 staining (brown) of the wild type EBs (B – a, d) representing nerve fibers while the *rybp*^{-/-} (B – e) and *Tg^{rybp}* cells (B – f) shows reduced density of nerve fibers (black arrows). scale bar equals 100 µm, magnification: 15x

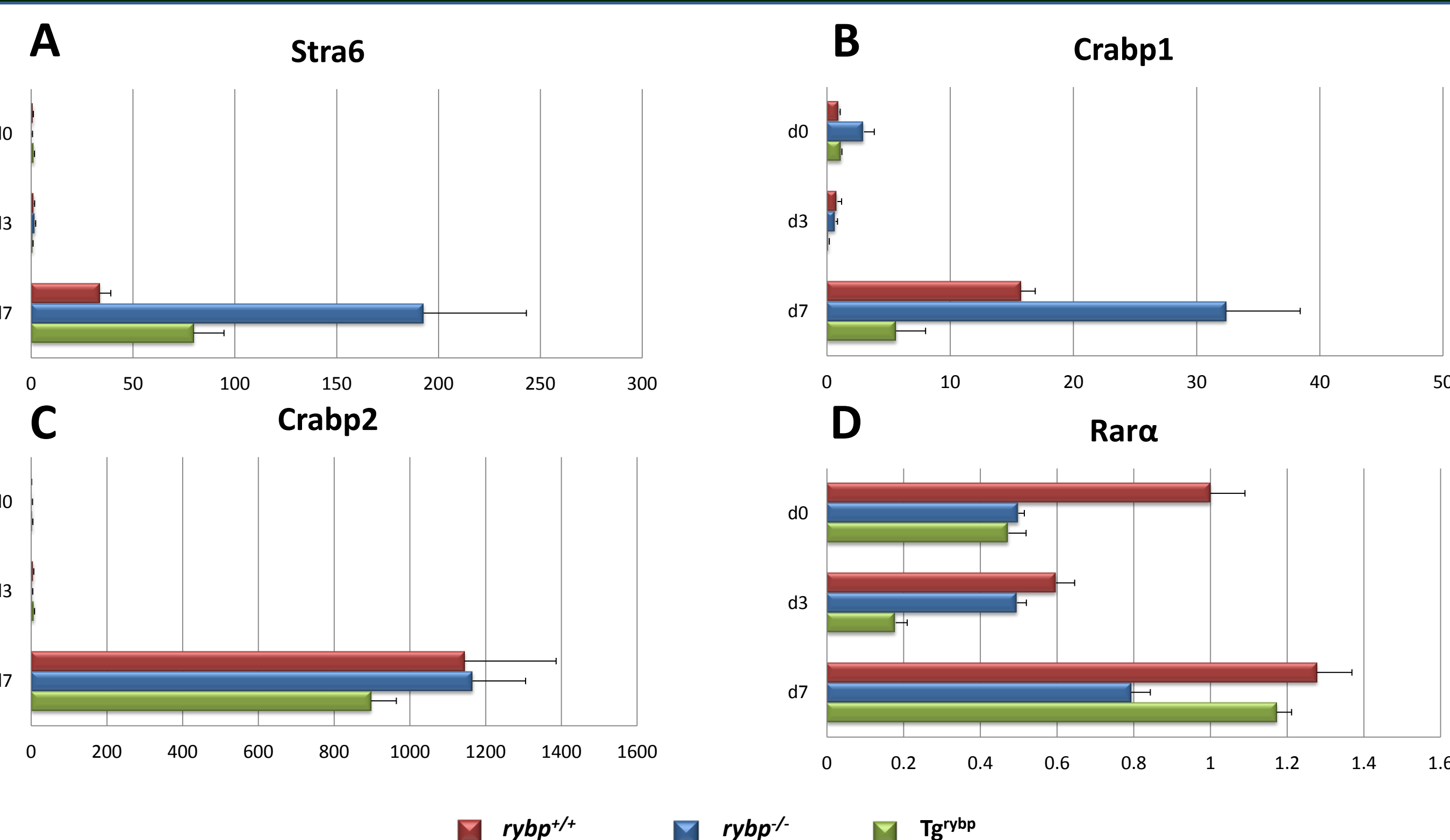


Figure 6 – Graphic representation of relative gene expression changes of the RA pathway members Stra6, Crabp1, -2 and Rara

By d7 the expression of Stra6 and Crabp1 is elevated in *rybp*^{-/-} cells compared to the controls. There is no difference in Crabp2 and Rarα expression in different cell lines. n = 3, p<0.05, Error bars represent standard deviation.

Discussion

Here we show, that altered Rybp dosage influences the expression of the RA pathway members (Stra6, Crabp1) and that *rybp* null cells are more sensitive to RA treatment than the wild type. Results from qRT-PCR experiments suggest that the expression of Crabp2 (Fig.6C) and Rarα (Fig.6D) is not influenced by the dosage of Rybp. It is more likely that elevated Stra6 expression (Fig.6A) caused increase in RA sensitivity of the *rybp*^{-/-} cells. Stra6 is overexpressed in some tumors and its upregulation causes defects in eye development, similarly to the phenotype observed in the *in vivo* *rybp*^{-/-} mouse model which also has coloboma of the eye. The fact that the phenotypes coincide may indicate a possible interplay between the two genes. Crabp1 gene expression analysis (Fig.6B) suggests that Rybp downregulates Crabp1 expression. In agreement with this observation overexpression of Crabp1 caused axial defects and coloboma in mouse models as well.

However ES cells from all three genotypes (*rybp*^{+/-}, *rybp*^{-/-} and *Tg^{rybp}*) formed EBs and that primitive ectodermal and neuroepithelial layers were also formed, lack of Rybp resulted accelerated neuroepithelium formation. This can be an excessive response for RA (Fig.5Ae). This is in agreement with our *in vivo* observations when reduced level of Rybp manifest midbrain overgrowth in the *rybp* heterozygous mice (Pirty, 2005).

Previously we have demonstrated that the *rybp*^{-/-} mutant mouse ES cell line displays inhibition of axon formation that reflects a delay in axon outgrowth and a reduced rate of elongation (Kovacs, 2012). Proper axonal and dendritic development requires a complex interplay of transcription factors, cell adhesion molecules and microtubule formation. Tubb3 is one of the factors which is important in this process and its level is barely detectable in the *rybp*^{-/-} and *Tg^{rybp}* lines in comparison to the wild type (Fig.5Bd,e,f). This suggests that the normal level of Rybp is also important for the proper development of the axonal or dendritic growth or pathfinding.

The increased RA sensitivity of the cells in lack of Rybp may responsible for the increased neuroepithelium formation, while in case of Rybp overexpression the genes stay repressed.

Future plans include further investigation of the RA pathway members in order to reveal the possible interactions between Rybp and RA pathway members.

Acknowledgments

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