

TRBP maintains mammalian embryonic neural stem cell properties by enhancing the Notch signaling pathway as a novel transcriptional coactivator

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Key Words: TRBP, Notch, Embryonic neural stem cells, Transcriptional activation, Central nervous system development

Summary

In the developing mammalian brain, TRBP regulates neural stem cell characteristics by stabilization of the Notch transcriptional coactivation complex conferring efficient Notch target gene expression.

ABSTRACT

Transactivation response element RNA-binding protein (TRBP) is known to play important roles in human immunodeficiency virus (HIV) replication and microRNA biogenesis. However, recent studies implicate TRBP in a variety of biological processes as a mediator for cross-talk between signal transduction pathways. Here, we provide the first evidence that TRBP is required for efficient neurosphere formation, and expression of neural stem cell markers and Notch target genes in primary neural progenitor cells *in vitro*. Consistent with this, introduction of TRBP into the mouse embryonic brain *in utero* increased the fraction of cells expressing Sox2 in the ventricular zone (VZ). We also show TRBP physically interacts with the Notch transcriptional coactivation complex through C promoter binding factor 1 (CBF1) and strengthens the association between the Notch intracellular domain (NICD) and CBF1, resulting in increased NICD recruitment to the promoter region of a Notch target gene. Our data indicate that TRBP is a novel transcriptional coactivator of the Notch signaling pathway playing an important role in neural stem cell regulation during mammalian brain development.

INTRODUCTION

Mammalian brain development requires tightly regulated-spatiotemporal control of neural stem cell proliferation and differentiation for proper neural cell composition, laminar organization and size of a mature brain (Rakic, 1995; Breunig et al., 2011). This regulation is achieved by numerous cellular signaling pathways triggered by various cues such as secretory factors and cell surface ligands (Yoon and Gaiano, 2005; Kohwi and Doe, 2013).

Transactivation response element RNA-binding protein (TRBP) was initially identified as a human immunodeficiency virus type 1 (HIV-1) TAR RNA binding partner (Gatignol et al., 1991; Dorin et al., 2003), and received much attention because of its activity in enhancing the catalytic activity of Dicer, a key regulator in microRNA biogenesis (Chendrimada et al., 2005; Haase et al., 2005). However, more recently, TRBP has been proposed to have miRNA-independent functions in various cellular events including cell growth, oncogenesis, and antiviral response by mediating cross-talk between signal transduction pathways (Daniels and Gatignol, 2012). Interestingly, TRBP was detected in the ventricular zone of the mammalian embryonic brain at a significant level (www.eurexpress.org), but its role there has not yet been determined.

Notch controls crucial functions in many tissues in adults as well as various developmental stages (Yoon and Gaiano, 2005). In the developing mammalian central nervous system, Notch controls maintenance and cell fate choices of neural stem cells (Yoon et al., 2004). In this study, we establish an unexpected functional link

between TRBP and the Notch signaling pathway in the regulation of neural stem cell characteristics during mammalian brain development.

RESULTS AND DISCUSSION

We first confirmed TRBP expression in the mouse embryonic brain by *in situ* hybridization and immunofluorescence staining. Both techniques revealed TRBP expression in the germinal zones lining the lateral ventricles in the embryonic age 13.5 (E13.5) forebrain, implying a potential role in neural progenitor populations (Fig. 1A, B). To test the functions of TRBP in embryonic neural progenitor cells *in vitro*, we began with neurospheres (Singec et al., 2006) isolated after transducing E14.5 neural progenitors with a TRBP-expressing retroviral vector MSIG (Han et al., 2015). Expression of TRBP gave rise to an increase in the number and size of neurospheres compared to control virus (Fig. 1C), suggesting that TRBP enhances the self-renewal capacity of neural progenitor cells *in vitro*. Increased expression of neural stem cell markers, Sox2 and Pax6 (Ellis et al., 2004; Pevny and Nicolis, 2010), supported this idea (Fig. 1E, G). Consistent with these results, TRBP knockdown by short hairpin RNA reduced the size and frequency of neurospheres, and expression of Sox2 and Pax6 (Fig. 1D, F, G). Providing important confirmation of our results in an *in vivo* context, we found that cells expressing the TRBP vector were more likely to express Sox2 and be located in the ventricular/subventricular zone (VZ/SVZ) after *in utero* electroporation (Fig. 1H, I). Interestingly, we also noted that TRBP increased astrocyte production at the expense of neurogenesis under differentiating conditions

in vitro (Fig. 1J, K); this is reminiscent of Notch effects in the embryonic central nervous system (Yoon and Gaiano, 2005; Jang et al., 2014).

These results led us to examine whether TRBP regulates the Notch signaling pathway. Indeed, in primary neural progenitor cells, TRBP increased mRNA expression levels of the Notch targets *Hes1* and *Hes5* to levels comparable to those driven by the Notch intracellular domain (NICD, constitutively active form of Notch) (Yang et al., 2011), and levels of *Hey1* mRNA expression to a level corresponding to roughly 50% of NICD-stimulated levels (Fig. 2A-C). TRBP shRNA-infected cells had a more than 50% reduction in the levels of *Hes1*, *Hes5*, and *Hey1* mRNAs (Fig. 2D-F). However, unlike the qRT-PCR results using primary neural progenitor cells, TRBP alone did not activate the Notch reporter driven by a C promoter binding factor 1 (CBF1)-responsive element (four CBF1-binding sites and the basal SV40 promoter) in NIH3T3 cells (Fig. 2G), having undetectable levels of Notch activity. Instead, TRBP dramatically increased promoter activity only when NICD was present, suggesting that upregulation of Notch target gene transcription by TRBP occurs at the promoter level, and is NICD-dependent. Moreover, co-expression of a dominant negative form of Mastermind-like 1 (dnMAML1), a key component of the Notch coactivation complex (Wu et al., 2000; Weng et al., 2003), disrupted the effects of TRBP (Fig 2G, I, J), indicating that TRBP action requires core Notch coactivational machinery. qRT-PCR revealed that gene expression levels of Notch coactivational complex members were not changed by the gain- or loss-of-function of TRBP in primary neural progenitor cells (supplementary material Fig. S1A, B), suggesting that the enhanced

Notch target promoter activation by TRBP was not due to the increased gene expression of a coactivational complex member. In primary neural progenitor cells, forced expression of TRBP alone increased Notch-dependent promoter activity presumably because of endogenous NICD presence in these cells, and co-transfection with NICD-expressing vector enhanced activity even further (Fig. 2H), consistent with Fig. 2A-C. Facilitated *in vivo* activation of the Hes5 promoter was also observed when a Discosoma red (DsRed) reporter driven by the Hes5 promoter was introduced together with the TRBP plasmid into the brain (supplementary material Fig. S2).

Since the most well-known function of TRBP is to enhance Dicer activity, we tested if Dicer was involved in TRBP enhancement of Notch pathway activation by generating a TRBP mutant lacking Dicer-binding ability (TRPB Δ C) (Fig. 2K) based on a previous report (Daniels et al., 2009). This TRBP mutant still efficiently increased Hes5 mRNA production and Sox2 protein levels (supplementary material Fig. S3A, B) in neural progenitor cells *in vitro*, and induced cell localization to the VZ/SVZ *in vivo* (Fig. 2L, M) at wild-type levels, strongly indicating that TRBP enhancement of Notch pathway activation is Dicer-independent. Previous studies also support our observations: the expression of Hes genes was not altered in Dicer-ablated embryonic brains (Peng et al., 2012), and neural progenitors lacking Dicer did not precociously exit the ventricular zone and still retained progenitor-marker expression *in vivo* (Knuckles et al., 2012).

Since TRBP is a nuclear protein and simultaneously increases the

transcription of several Notch target genes, one possible mechanism explaining these observations may involve direct modulation of transcriptional machinery. To test this hypothesis, we used coimmunoprecipitation (co-IP) assays with the HEK 293T cell line to explore the potential interactions between TRBP and the Notch transcriptional activation complex. Myc-tagged NICD coimmunoprecipitated with TRBP-HA protein, whereas no band was detected from GFP-HA-transfected cells (Fig. 3A). Likewise, clear association of TRBP with CBF1 or MAML1 was also observed (Fig. 3B, C). TRBP coimmunoprecipitated well with CBF1 in RNase-treated samples suggesting that TRBP interacts with Notch components in an RNA-independent manner (supplementary material Fig. S4A, B).

Consistent with the co-IP results, a chromatin immunoprecipitation (ChIP) assay using chromatin extracts derived from E14.5 primary neural progenitor cells and anti-TRBP antibody revealed that endogenous TRBP protein is recruited to the genomic Hes5 promoter region (Fig. 3D). Furthermore, the glutathione S-transferase (GST)-tagged CBF1 protein was able to pull down TRBP in a cell-free *in vitro* binding assay (Fig. 3E), suggesting that CBF1 is a direct binding target of TRBP. The interaction between endogenous TRBP and CBF1 was also confirmed in primary mouse neural progenitors and HeLa cells by co-IP assays with anti-TRBP and anti-CBF1 antibodies (Fig. 3F, G).

Finally, we examined the functional consequences of TRBP association with the Notch transactivational complex using the HEK 293T cell line. Overexpression of TRBP caused an increase in association between CBF1 and NICD up to 2.5 fold (Fig.

4A), and a mutant TRBP protein deficient in dsRNA binding domains and binding activity to CBF1 (TRBPM) lost this ability (Fig. 4B, C). Furthermore, this interaction was significantly weakened by TRBP knockdown (Fig. 4D). Considering the sizable amount of residual TRBP protein in TRBP shRNA-expressing cells, this reduction rate strongly indicates that TRBP plays an important role in stabilizing the Notch coactivational complex at physiological levels.

The importance of TRBP interaction with CBF1 was further confirmed *in vivo*. Cell exit from the VZ/SVZ was prominent 2 days post-electroporation of a shRNA vector against TRBP at E13.5, and this change was reversed by wild-type TRBP and not by TRBPM (Fig. 4E, F) indicating that TRBP-induced augmentation of neural stem cell properties is strictly CBF1-binding dependent.

Because both TRBP and CBF1 are nuclear, and are ubiquitously expressed in many types of tissues (Lu and Lux, 1996; Daniels and Gagnon, 2012), it is plausible to hypothesize that TRBP may be a constitutive component of the Notch coactivation complex. Our data showing interaction between TRBP and CBF1 in non-neural human HeLa cells (Fig. 3F) provides support for this idea. Examination of the role of TRBP in cellular events such as oncogenesis or hematopoiesis in which Notch signaling plays a pivotal role would yield insight into the potential ubiquitous importance of this Notch coactivation complex member.

MATERIALS AND METHODS

Animals

All animal studies were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee at the Sungkyunkwan University (SKKUIACUC-2016-04-0005-2). Timed-pregnant CD1 mice were obtained from Koatech (Korea) and used for neural progenitor cell preparation and *in utero* electroporation.

In situ hybridization

In situ hybridization was conducted according to standard protocols (Siegenthaler et al., 2009). Digoxigenin-labeled sense and antisense riboprobes were generated from pGEM-T Easy vectors (Promega) containing the cDNA sequences corresponding to residues 1-623 of mouse TRBP.

Coimmunoprecipitation (co-IP)

For immunoprecipitations of tagged proteins, transfected HEK 293T cells obtained from the American Type Culture Collection (ATCC, Manassas, VA) were lysed in immunoprecipitation lysis buffer [50 mM pH 7.5 Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA] with protease inhibitor (Sigma). Total proteins were incubated with primary antibody against a tag [mouse anti-Myc (Santa Cruz Biotechnology, sc-40X; 1:1,000); mouse anti-HA (Abcam, ab16918; 1:500); mouse anti-

FLAG (Sigma, F1804; 1:500)] and protein G-conjugated beads (Invitrogen) at 4°C overnight. Beads were washed 3 times with lysis buffer and 2× sample buffer was added. Samples were then boiled for 10 min, and beads were removed by centrifuging at 17,000 × g for 30 min. Proteins were resolved by SDS-PAGE and subjected to immunoblotting using an anti-tag antibody. The NIH Image J software (<http://rsb.info.nih.gov/ij/>) was used to quantify band intensity of the immunoblot. Immunoprecipitations of endogenous TRBP with anti-TRBP antibody (a kind gift from Dr. V. N. Kim, Seoul National University, Seoul, Korea) using mouse embryonic neural progenitors or HeLa cells, and subsequent immunoblotting with anti-CBF1 antibody (Cosmo Bio, ZRBP2; 1:100) were performed in a similar manner.

GST pull-down assays

The TRBP and CBF1 genes were cloned into the bacterial expression vector pGEX-4T1 (GE Healthcare Life Science) which has a glutathione S-transferase (GST) tag at the N-terminus and a thrombin cleavage site, and GST fusion proteins were expressed in bacterial BL21 strain cells by the IPTG induction method and purified as described (Lee et al., 2006). Recombinant TRBP protein without the GST tag was prepared by incubating GST-TRBP protein with 1 U/μl of thrombin (Sigma) at room temperature overnight, followed by inactivation with 1 mM PMSF. GST alone or GST-CBF1 protein coupled to glutathione-agarose beads (Sigma) were incubated with thrombin-cleaved TRBP protein at 4°C overnight. The mixtures were washed 3 times with PBS and eluted with glutathione elution buffer [50 mM Tris-HCl (pH8.0),

10 mM reduced glutathione (Sigma)]. The samples were then subjected to SDS-PAGE followed by Western blot analysis.

Statistical Analysis

Statistical tests were performed using SPSS (statistical product and service solution software, IBM) version 23. All data represent three or more independent experiments.

Acknowledgements

We thank Dr. V. N. Kim for the kind gift of anti-TRBP antibody.

Competing interests

The authors declare no competing financial interests.

Author contributions

S.-H.B., D.H., J.K., M.K., J.Y.C. and H.X.N. carried out experiments and analyzed data. K.Y. designed the study and wrote the manuscript with contribution from S.J.P.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (2015R1A2A2A01005687)

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Figures

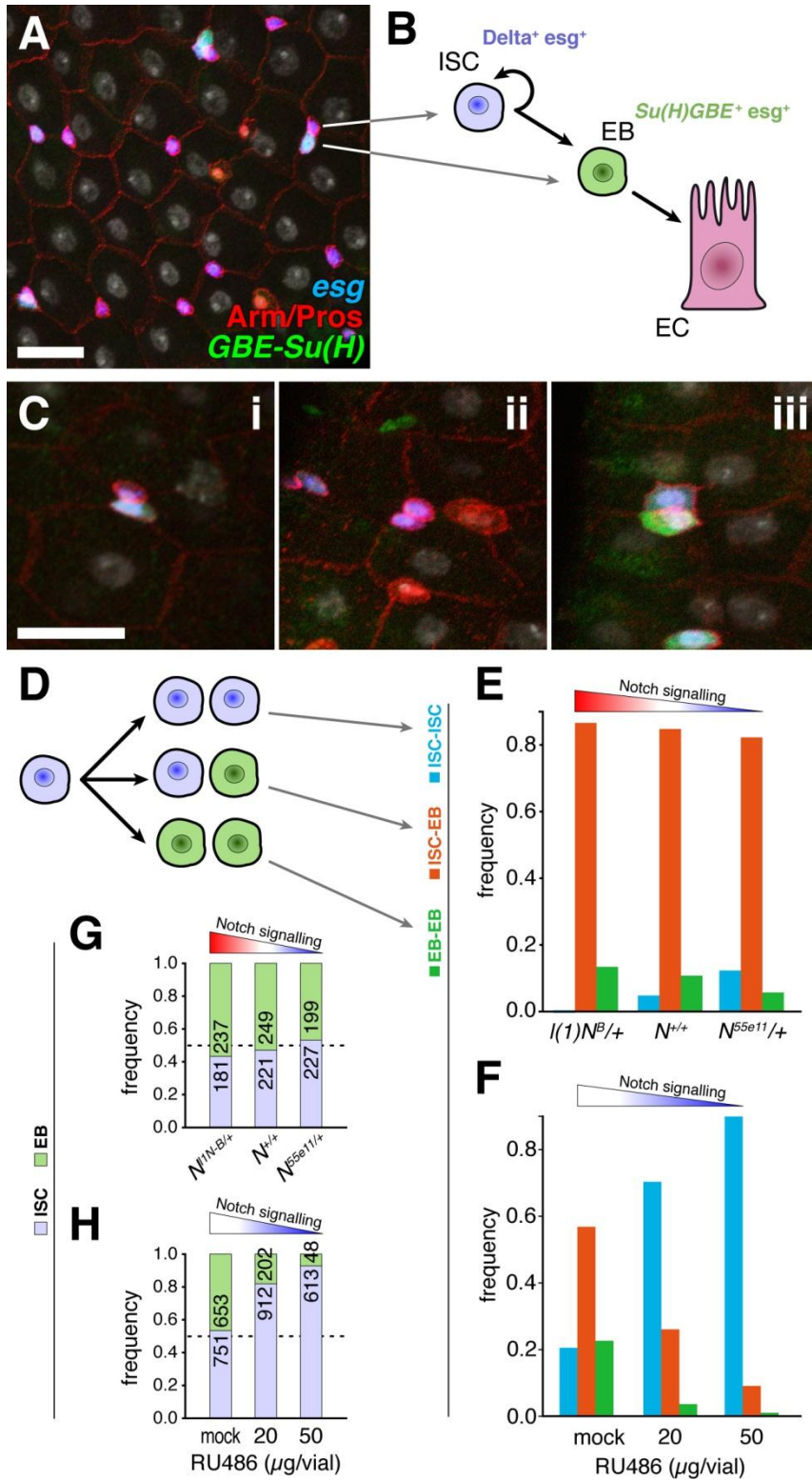


Fig. 1. TRBP enhances embryonic neural stem cell properties. TRBP gene expression pattern in the E13.5 mouse forebrain was determined by (A) *in situ* hybridization and (B) immunofluorescence after antigen retrieval using proteinase K digestion. Neurosphere assay using mouse E14.5 primary neural progenitors transduced with a retroviral vector expressing (C) TRBP or (D) short hairpin RNA specific to TRBP (shTRBP). Western blot analysis of neural progenitor cell lysates infected with (E) TRBP- or (F) shTRBP-expressing retrovirus for Pax6 and Sox2 proteins. Quantification is shown in (G). (H) Double-immunolabeling of E15.5 brain sections electroporated *in utero* with TRBP-expressing plasmid at E13.5 using anti-GFP (reporter gene, green) and Sox2 (red) antibodies. Quantification is shown in (I). (J) Representative immunostaining using anti-GFP (green) together with anti- β III-tubulin (clone TuJ1) (neuron, red) or GFAP (astrocyte, red) antibodies after *in vitro* differentiation of E14.5 neural progenitor cells. Quantification is shown in (K). Scale bars, 200 μ m for (A), and 50 μ m for (B), (H), and (J). CP, cortical plate; Cx, cortex; GE, ganglionic eminence; IZ, intermediate zone; LV, lateral ventricles; MZ, marginal zone; SVZ, subventricular zone; VZ, ventricular zone. All error bars represent s.d. Student's *t*-test was used to determine statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

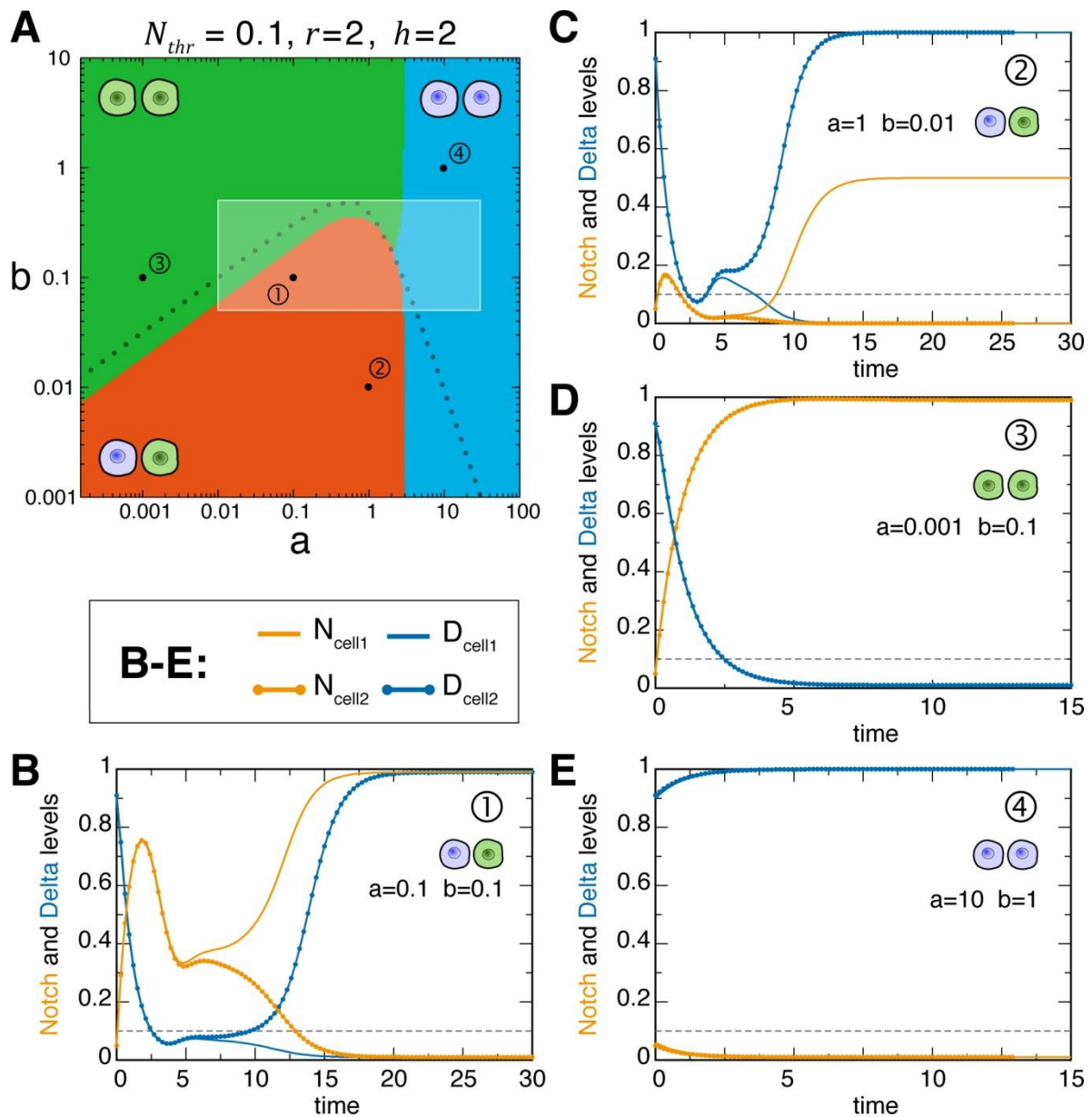


Fig. 2. TRBP increases Notch-dependent transcription. mRNA expression levels of each indicated Notch target gene measured by qRT-PCR, two days after E14.5 primary neural progenitor cells were transduced with retroviral vectors expressing (A-C) TRBP or (D-F) shTRBP. Luciferase assay to detect activation of a Notch reporter consisting of firefly luciferase fused to a CBF1-responsive element. (G) NIH3T3 or (H) E14.5 primary neural progenitor cells were transfected with a Notch reporter and the indicated expression vectors. Cells were harvested at 2 days

posttransfection for luciferase assays. (I, L) Anti-GFP immunolabeling of E15.5 brain sections electroporated *in utero* with the indicated expression vectors at E13.5. (J, M) Quantification of Fig. I and L, respectively. (K) Co-IP assays using HEK 293T cells show that a TRBP mutant (TRBP Δ C) is deficient in binding to Dicer. Scale bars, 50 μ m. Error bars represent s.d. Student's *t*-test was used to determine statistical significance. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

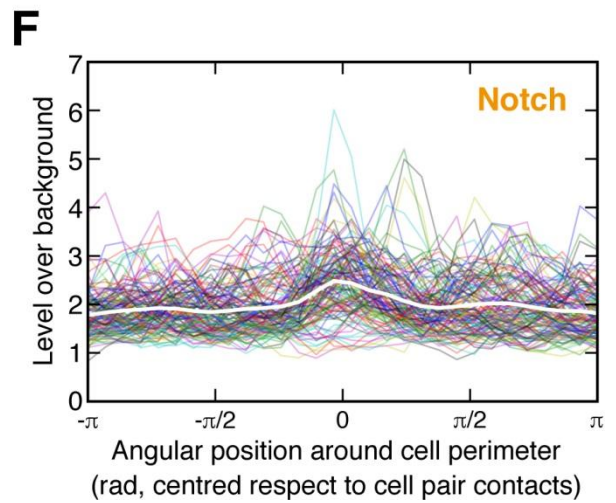
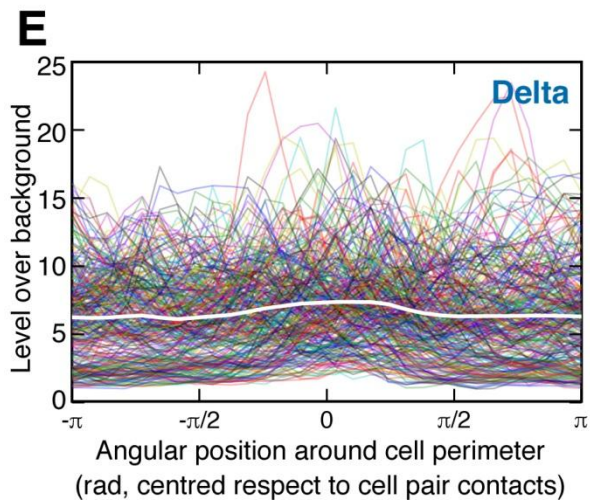
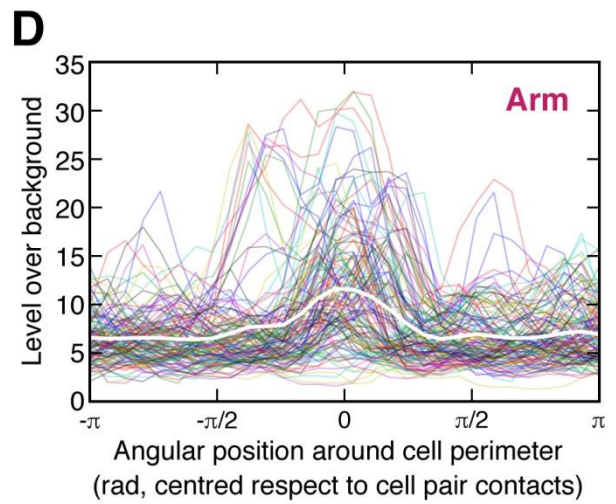
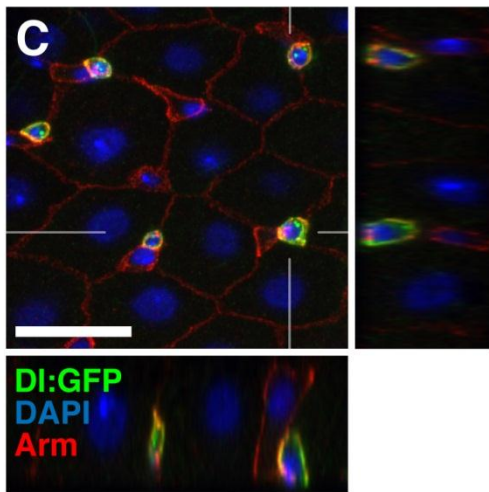
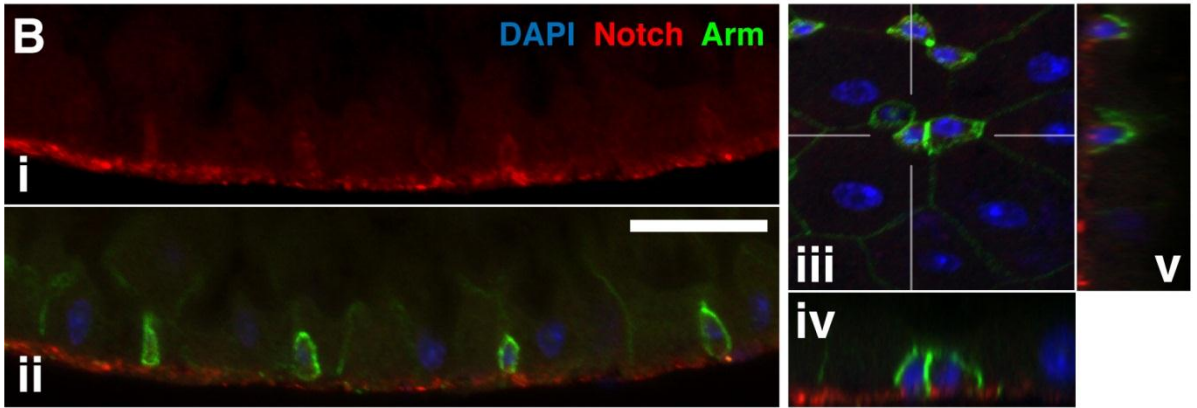
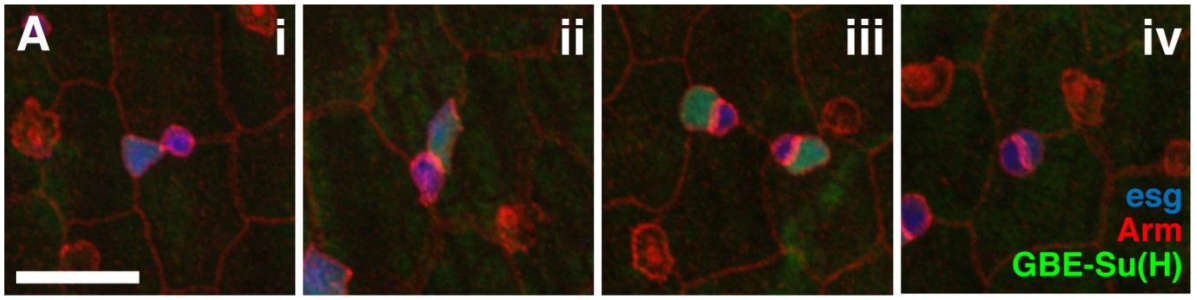


Fig. 3. TRBP interacts with the Notch transcriptional coactivation complex.

Representative Western blots showing coimmunoprecipitation (co-IP) of TRBP with each component of the Notch coactivational complex using 293T cell line. (A) NICD, (B) CBF1, and (C) MAML1. (D) Chromatin immunoprecipitation (ChIP) assay using primary neural progenitor chromatin extracts and anti-TRBP antibody, and subsequent real time PCR analysis for the genomic Hes5 and GAPDH promoter region. (E) GST pull-down assay using *Escherichia coli*-expressed recombinant GST-CBF1 and TRBP proteins. (F) Co-IP assays were performed with anti-TRBP and anti-CBF1 antibodies to show endogenous interactions between TRBP and CBF1 in primary mouse progenitors and HeLa cells. Quantification of neural progenitor results is shown in (G). Error bars represent s.d. Student's *t*-test was used to determine statistical significance. *** $P < 0.001$.

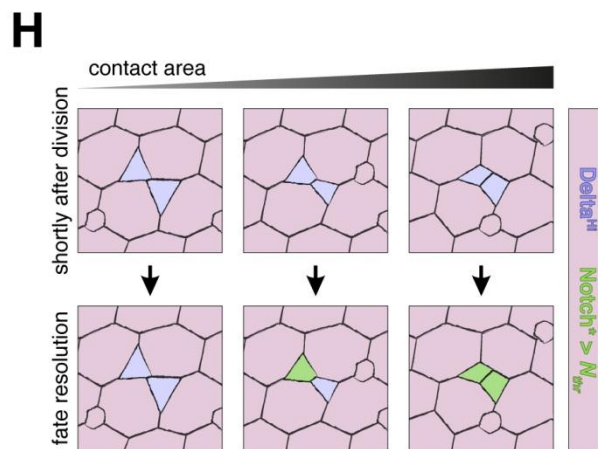
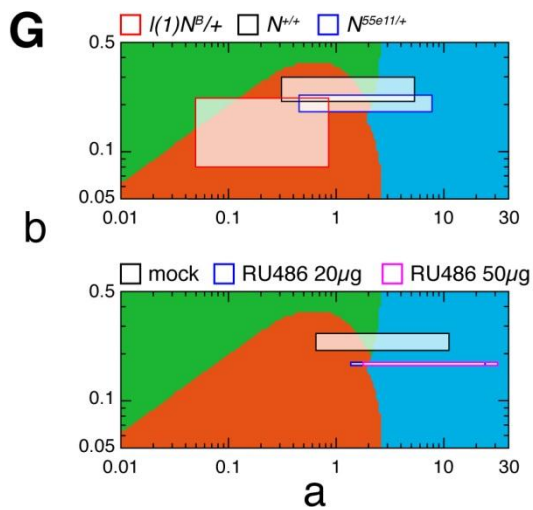
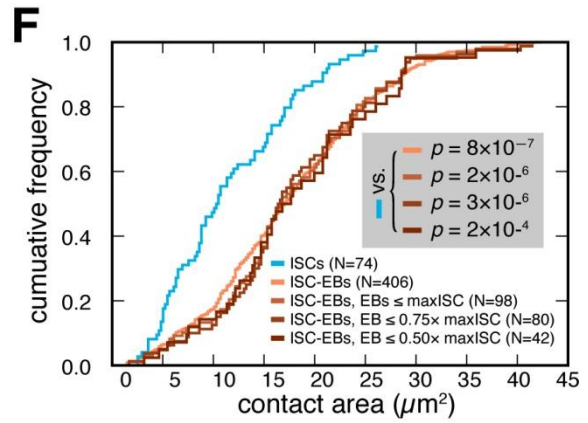
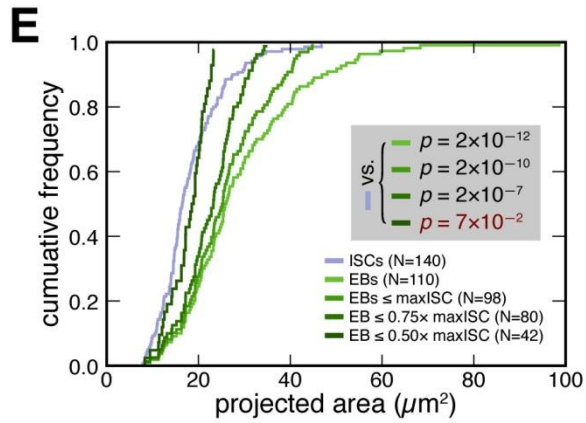
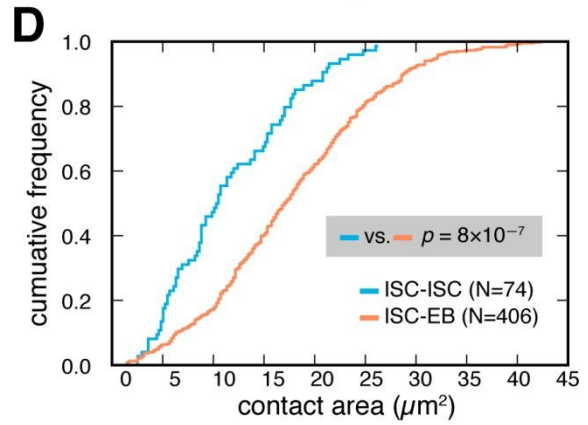
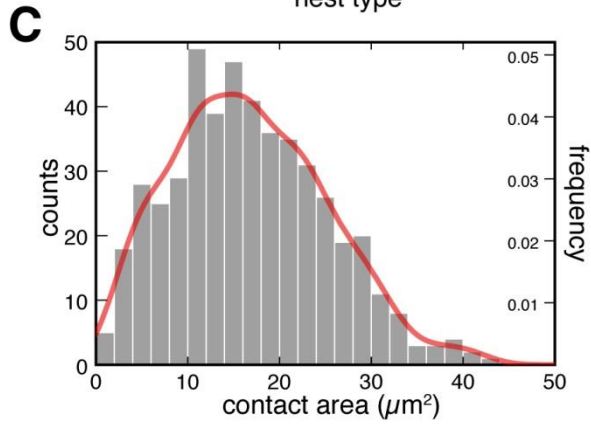
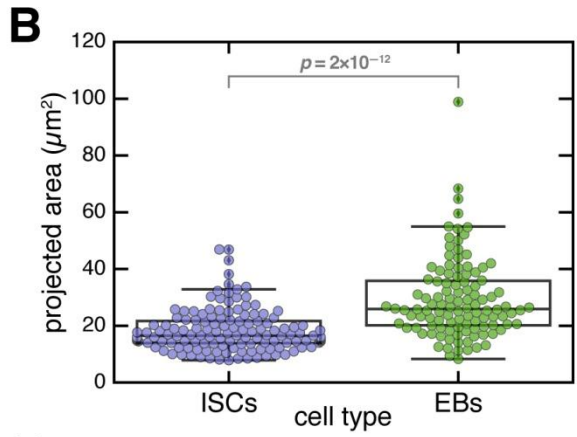
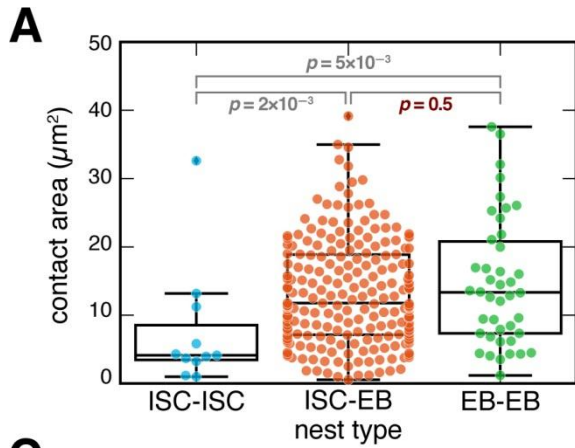


Fig. 4. TRBP reinforces association between NICD and CBF1. The binding affinity between NICD and CBF1 upon (A) overexpression or (D) knockdown of TRBP was examined by co-IP assays using the HEK 293T cell line. Coimmunoprecipitated CBF1-HA protein levels were normalized to CBF1-HA protein levels in the respective whole cell lysates. Co-IP assays show that a TRBP mutant (TRBPM) lacking dsRNA binding domains and deficient in binding to CBF1 (B) does not enhance the NICD/CBF1 interaction (C). TRBPM was identified through domain mapping experiments. (E) Immunolabeling of E15.5 brain sections electroporated *in utero* with the indicated expression vectors at E13.5 using anti-GFP and Alexa Fluor 555-conjugated secondary antibodies. Quantification is shown in (F). Scale bar, 50 μm . All error bars represent s.d. Student's *t*-test was used to determine statistical significance. * $P < 0.05$, ** $P < 0.01$.

Supplemental Information

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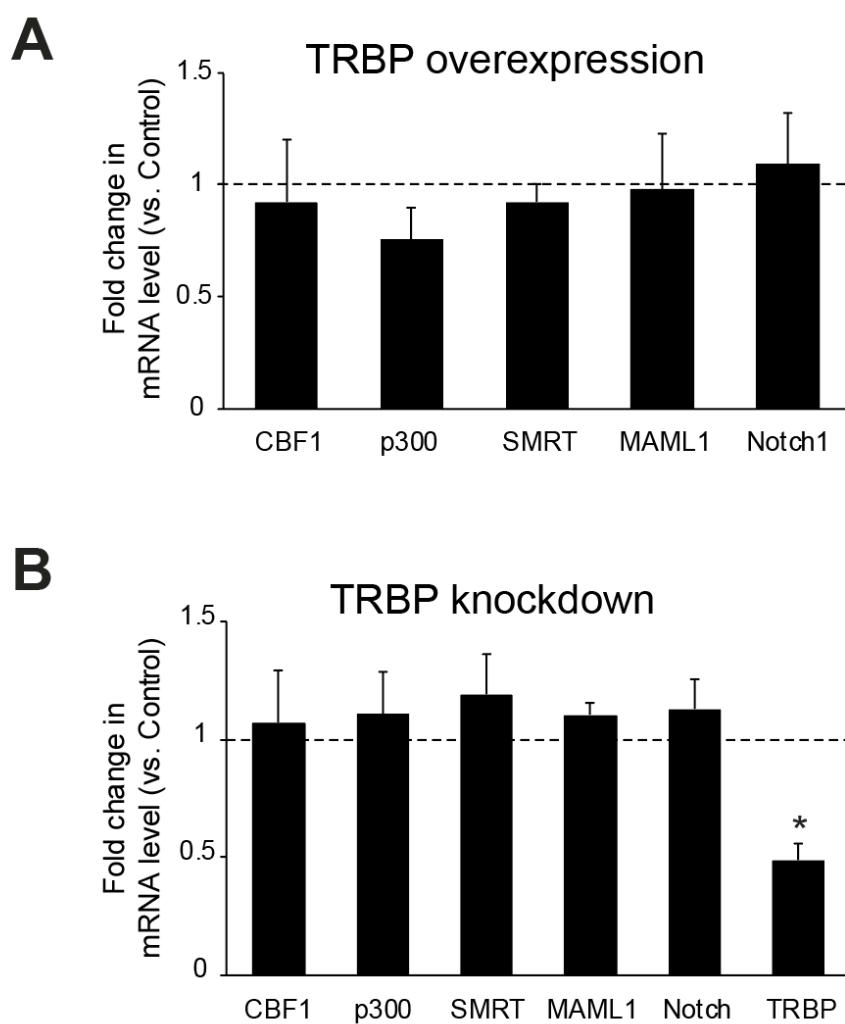


Figure S1. TRBP does not affect gene expression levels of Notch coactivational members. mRNA expression levels of each indicated Notch coactivational member were measured by qRT-PCR two days after E14.5 primary neural progenitor cells were transduced with retroviral vectors expressing (A) TRBP or (B) shRNA against TRBP. Error bars represent s.d. Student's *t*-test was used to determine statistical significance. * $P < 0.05$.

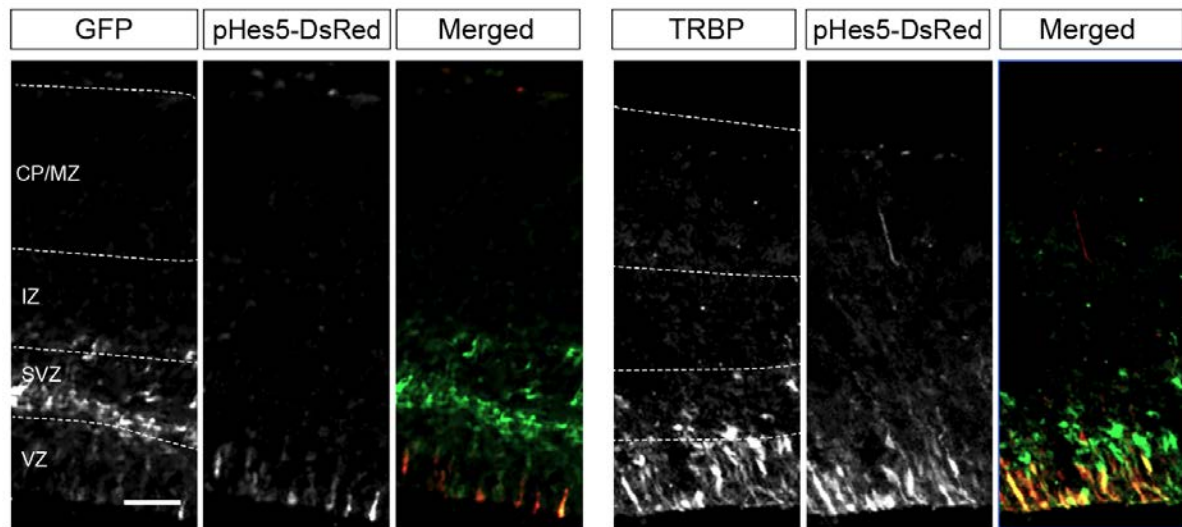


Figure S2. TRBP increases Hes5 promoter activity *in vivo*. Double immunolabeling of E14.5 brain sections electroporated *in utero* with the indicated plasmids at E13.5 using anti-GFP and anti-DsRed as primary antibodies, and Alexa dye-conjugated secondary antibodies to visualize effector gene expression (green) and Hes5 reporter activity (red). Scale bar, 50 μm .

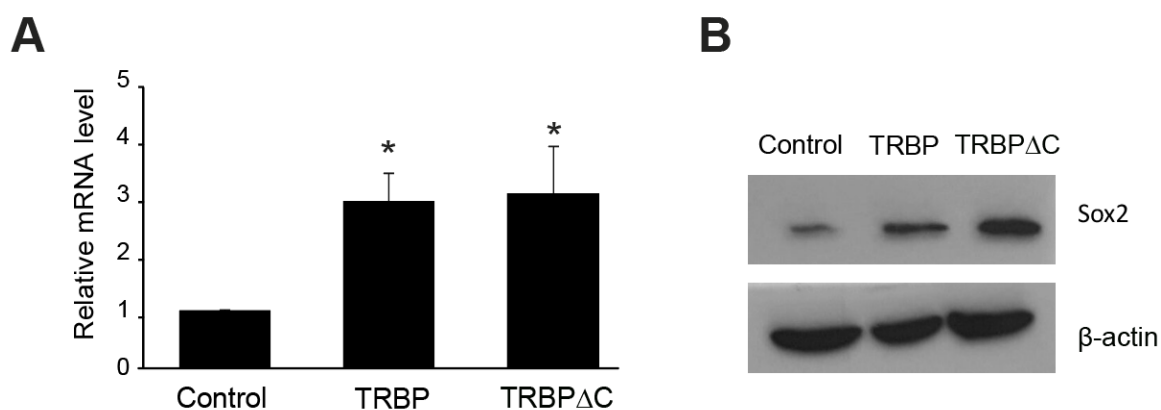


Figure S3. The Dicer-binding defective TRBP mutant behaves like wild-type

TRBP in neural progenitor cells. (A) Hes5 mRNA expression levels measured by qRT-PCR two days after E14.5 primary neural progenitor cells were transduced with retroviral vectors expressing TRBP or TRBP Δ C. (B) Western blot analysis of primary neural progenitor cell lysates infected with TRBP- or TRBP Δ C-expressing retrovirus for Sox2 proteins. Error bars represent s.d. Student's *t*-test was used to determine statistical significance. * $P < 0.05$.

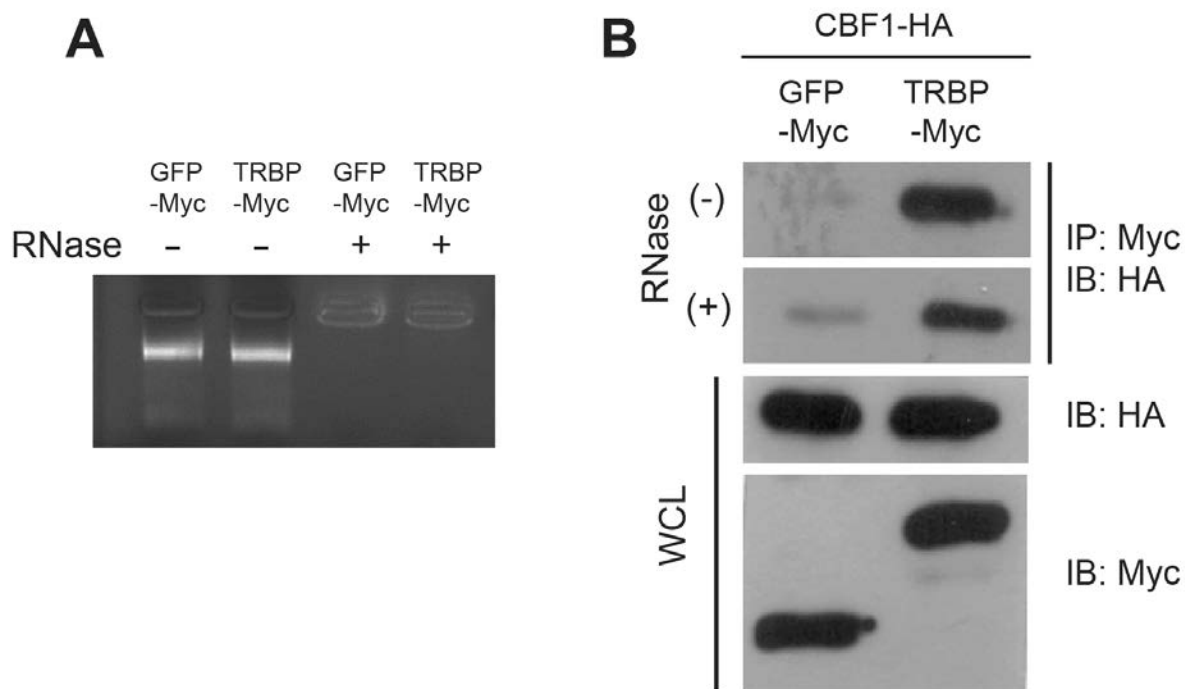


Figure S4. TRBP interacts with CBF1 in an RNA-independent manner. (A) Cellular RNA removal by RNase treatment of HEK 293T cell lysates prior to co-IP was assessed by agarose gel electrophoresis. (B) Co-IP assays were used to examine binding between TRBP and CBF1 with or without RNase pretreatment of cell lysates.

Supplemental Information

TRBP maintains mammalian embryonic neural stem cell properties by enhancing the Notch signaling pathway as a novel transcriptional coactivator

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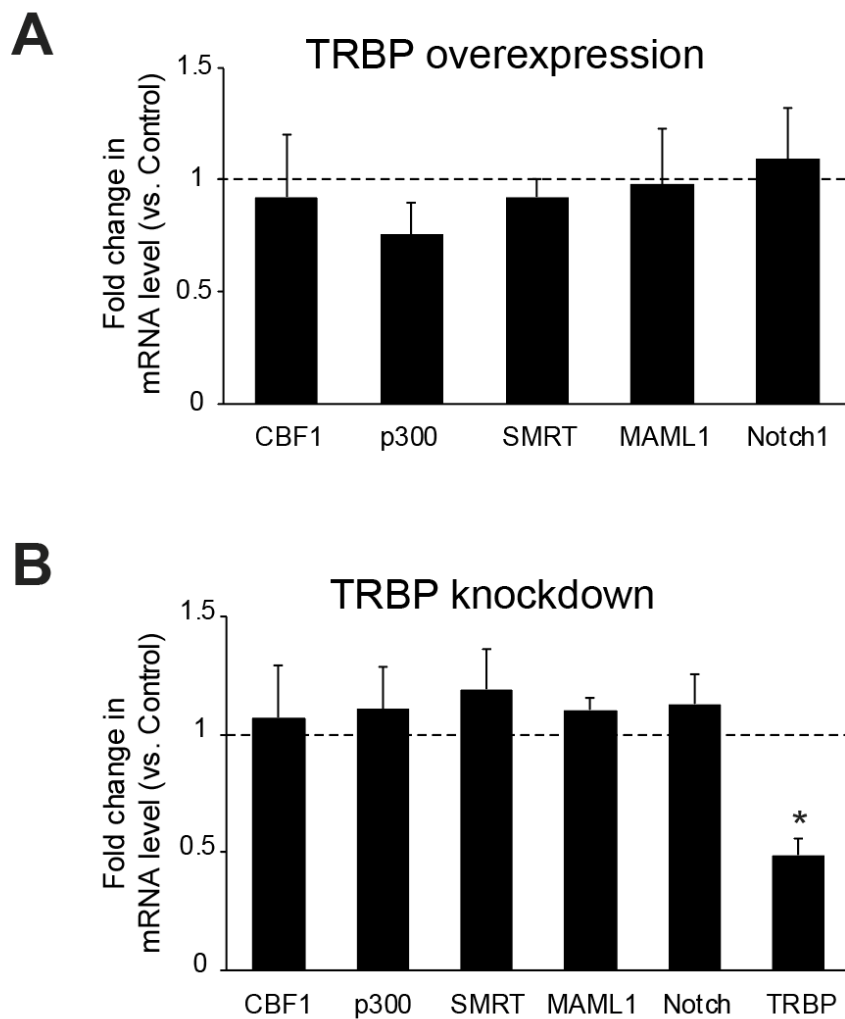


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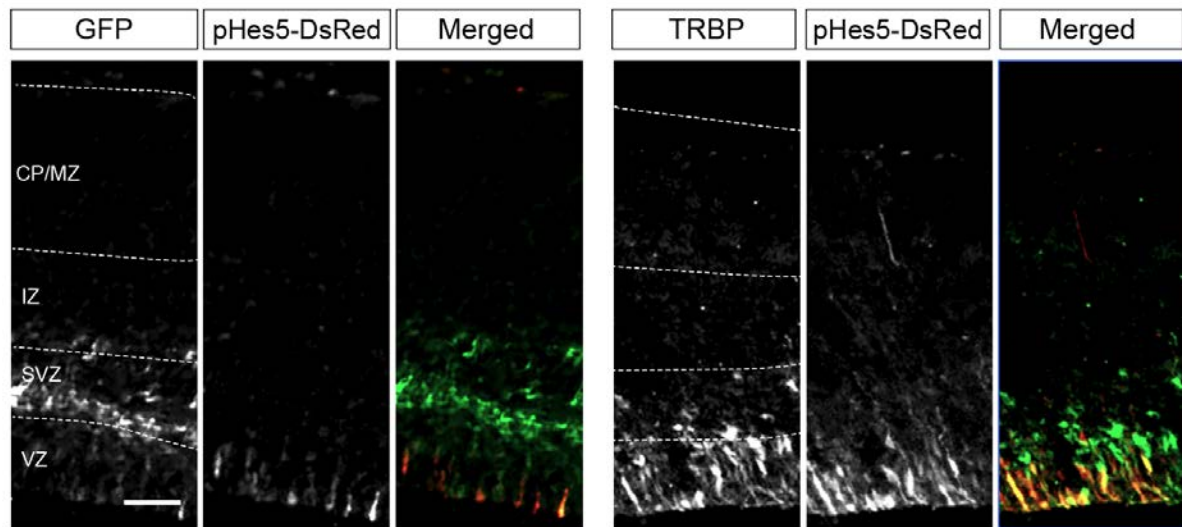


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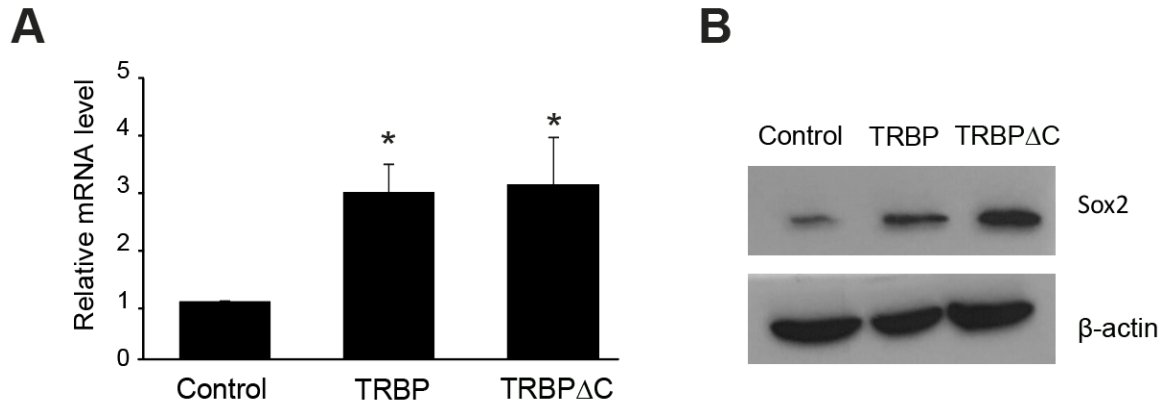


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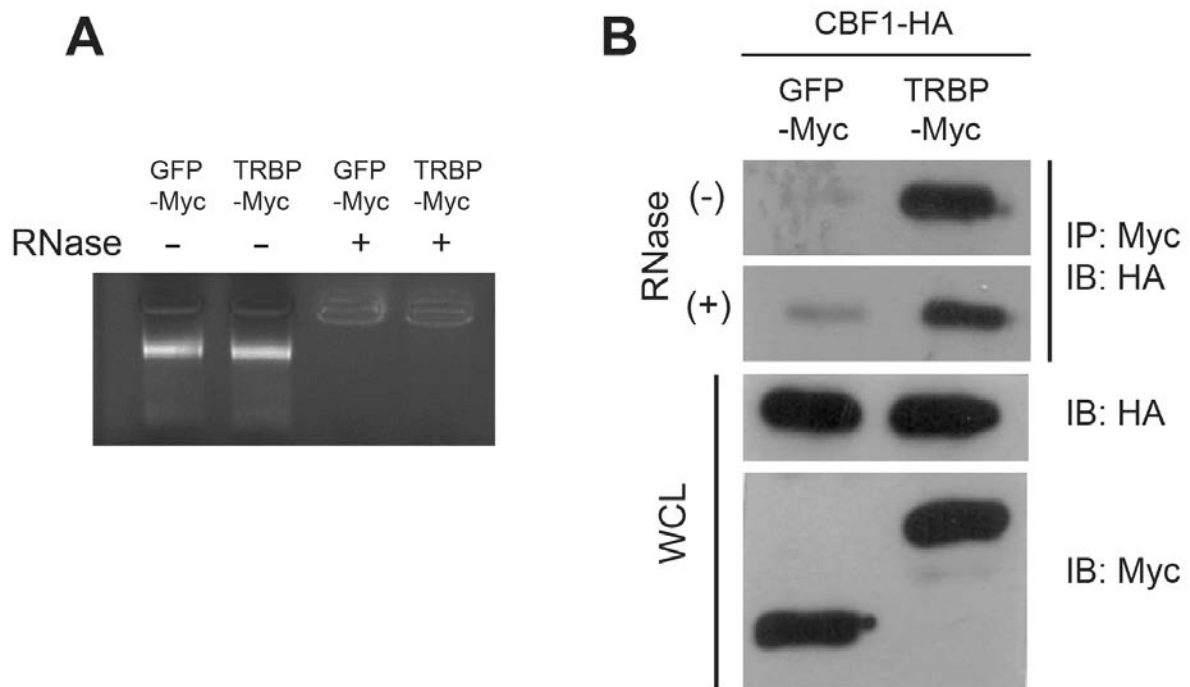


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