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Supplemental information

FTY720 requires vitamin B_{12}-TCN2-CD320 signaling in astrocytes to reduce disease in an animal model of multiple sclerosis

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Supplemental Data

FTY720 requires vitamin B_{12}-TCN2-CD320 signaling in astrocytes to reduce disease in an animal model of multiple sclerosis

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This file contains the following supplementary figures.

Fig. S1. snRNA-seq and CD320 identification.

Fig. S2. Analyses of B_{12}^{def} EAE mice and B_{12}^{free} astrocytes.

Fig. S3. FTY720 efficacy in B_{12}^{def} mice.

Fig. S4. CIR-based binding assay and CD320 internalization.

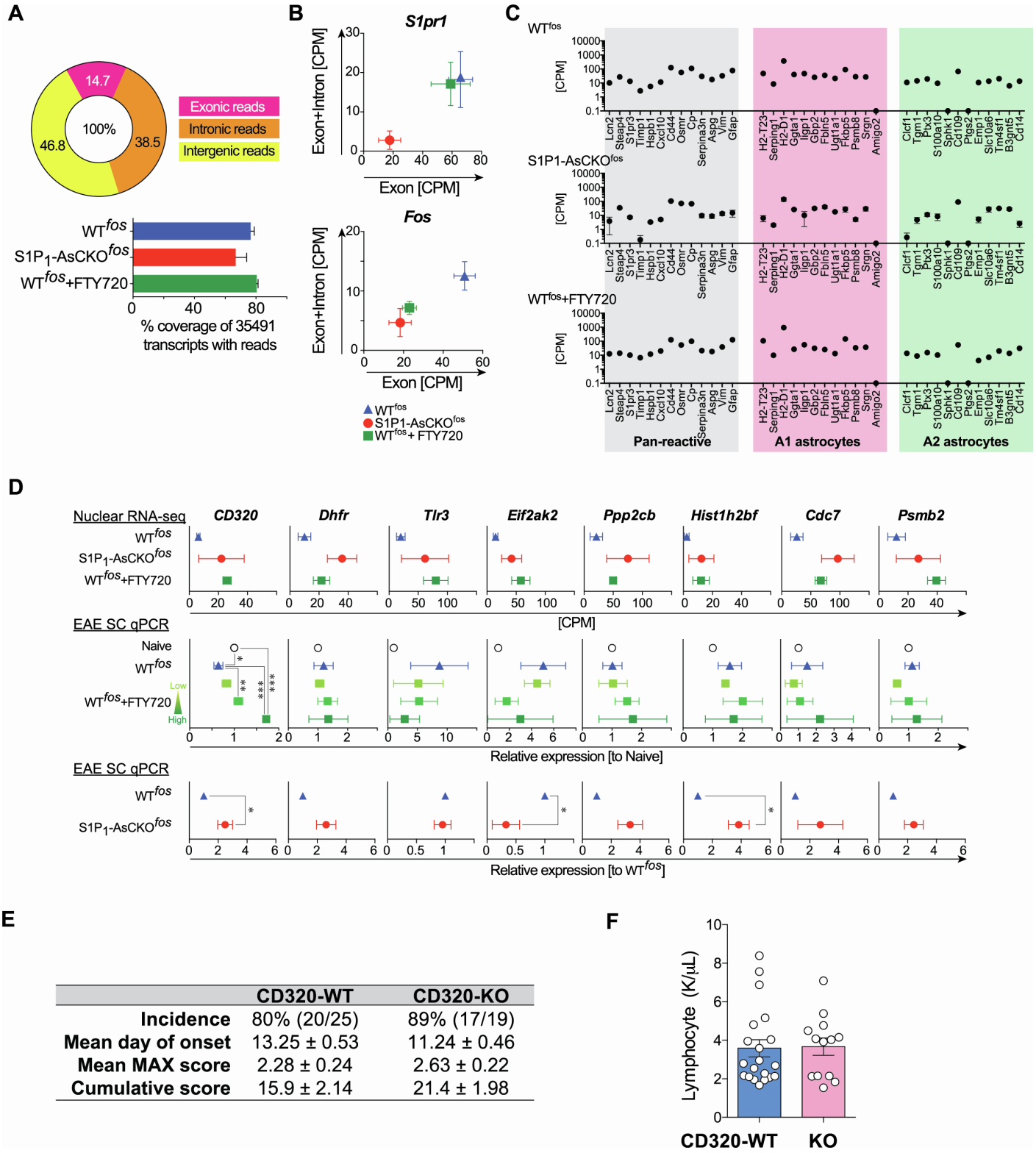
***Table S1.** DEGs of nuclear RNA-seq, corresponding to Fig. 1C

***Table S2.** Reactome pathway analysis[1] of Cluster I genes that were commonly up-regulated in $S1P_1$-$AsCKO^{fos}$ and FTY720-treated groups as compared to WT^{fos}.

***Table S3.** DEGs of astrocyte RNA-seq, corresponding to Fig. 2D.

***Table S4.** Primer sets.

*Supplementaly tables are provided in an Excel spreadsheet in a different file.



	CD320-WT	CD320-KO
Incidence	80% (20/25)	89% (17/19)
Mean day of onset	13.25 ± 0.53	11.24 ± 0.46
Mean MAX score	2.28 ± 0.24	2.63 ± 0.22
Cumulative score	15.9 ± 2.14	21.4 ± 1.98

Fig. S1. snRNA-seq and CD320 identification.

A, averaged percentage of exonic, intronic, and intergenic reads in nuclear RNA-seq. The percent of reads that mapped to exons and introns were uniquely characteristic of nuclear RNA, with an increased representation of intronic reads. Percent coverage of transcripts (mean ± SEM). **B**, the exonic and exonic+intronic reads of

S1pr1 were diminished only in S1P$_1$-AsCKO^{fos} when compared to WT^{fos} and WT^{fos}+FTY720, indicating that the DAPI$^+$NeuN$^-$GFP$^+$ nuclei were derived from astrocytes. The exonic and exonic+intronic reads of *fos* were diminished in both S1P$_1$-AsCKO^{fos} and WT^{fos}+FTY720, indicating less astrocytic activation in these mice. **C**, comparison between nuclear RNA-seq data vs. recently proposed Pan/A1/A2 reactive astrocyte-specific genes. Although there was no obvious A1/A2-skewing[2] in DAPI$^+$NeuN$^-$GFP$^+$ populations, more than 90% of reactive astrocyte-specific genes were detected. **D**, expression profile of 8 genes identified in the pathway analysis that correspond to **Fig. 1 C** (mean ± SEM, *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ by one-way ANOVA in FTY720 groups, and one sample t test in KO groups). **E**, clinical parameters of EAE in CD320-WT and CD320-KO mice. Composite data from three independent experiments are shown. Cumulative scores were the sum of clinical scores from days 0 to 20 (means ± SEM, * $p < 0.05$ by Mann-Whitney U test). **F**, peripheral lymphocyte numbers. Each circle represents a single animal (mean ± SEM).

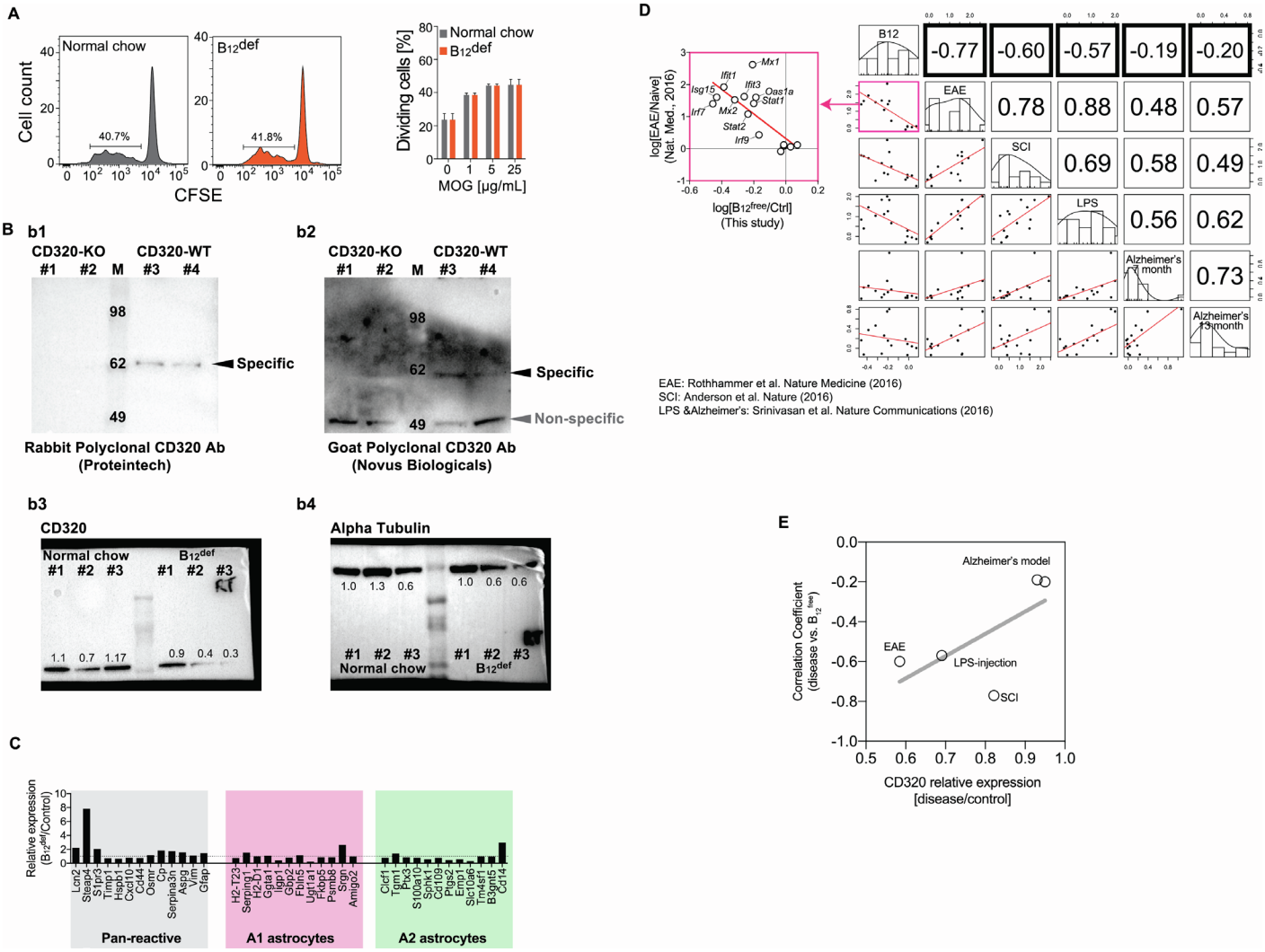


Fig. S2. Analyses of B_{12}^{def} EAE mice and B_{12}^{free} astrocytes.

A, T cell proliferation. Mononuclear cells were isolated from spleens of MOG35-55 immunized mice, and cultured in the presence or absence of MOG_{35-55} for three days. Representative histograms of carboxyfluorescein succinimidyl ester (CFSE) intensities of $CD3^+$ T cells are shown from two independent experiments. Percentage of dividing T cells is indicated. MOG-induced cell proliferation is comparable in B_{12}^{def} vs. controls (mean ± SEM, n = 3). **B**, Western blotting for CD320. (b1 and b2) Antibody specificities were assessed using proteins extracted from CD320-WT and CD320-KO SCs. The specific bands observed in CD320-WT disappeared in CD320-KO, indicating that both antibodies showed high specificity against mouse CD320. The calculated mass of CD320 is 27.7 kDa, but it is a heavily glycosylated protein[3]. (b3 and b4) CD320 expression was suppressed in EAE-induced B_{12}^{def} mice as compared with controls. Numbers indicate relative intensities. **C**, Comparison between astrocyte RNA-seq data vs. recently proposed Pan/A1/A2 reactive astrocyte specific genes. No obvious A1/A2-skewing [2] was observed in B_{12}^{def} astrocytes. **D**, Correlation matrix. Numbers indicate correlation coefficients. Correlation analyses of astrocytic IFN-I gene expression changes showed negative correlations between B_{12}^{free} (B_{12}-free astrocyte culture)vs. EAE[4], SC injury[5], and LPS-injected mice [6], but no correlation was found between B_{12}^{free} vs. Alzheimer's model mice[6]. **E**, correlation of CD320 fold changes vs. correlation coefficients between B_{12}^{free} (bold boxes in D) and indicated disease conditions. Moreover, these correlation coefficients were positively correlated with the extent of astrocytic Cd320 down-regulation in these diseases, suggesting an association of astrocytic IFN-I sensitivity with the B_{12}-CD320 pathway in neuroinflammation.

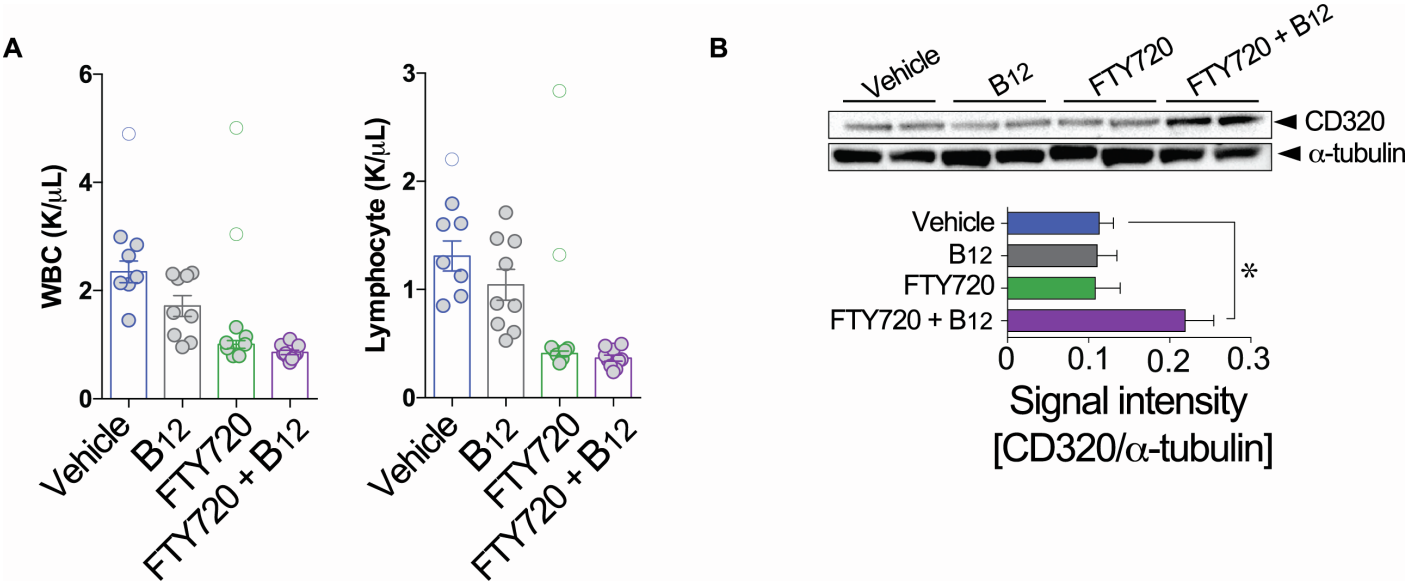


Fig. S3. FTY720 efficacy in B_{12}^{def} mice.

A, peripheral white blood cell (WBC) and lymphocyte counts in EAE mice treated with vehicle, B_{12}, FTY720, and FTY720+B_{12}. Lymphocyte trafficking effects of FTY720 appear to be intact for all experimental conditions examined. Each point represents a single animal. Some samples (open circles) were eliminated from the datasets based on Smirnov-Grubbs test before performing statistical analysis by Kruskal-Wallis test with Dunn's multiple comparisons test (mean ± SEM). **B**, Western blotting for CD320 in SCs of EAE mice at 50 days post immunization (dpi) and 25 days post treatment from 25 dpi. Signal intensity of CD320 was normalized by comparison to α-tubulin (mean ± SEM, n = 4 animals, * $p < 0.05$ by one-way ANOVA with Bonferroni's multiple comparisons test).

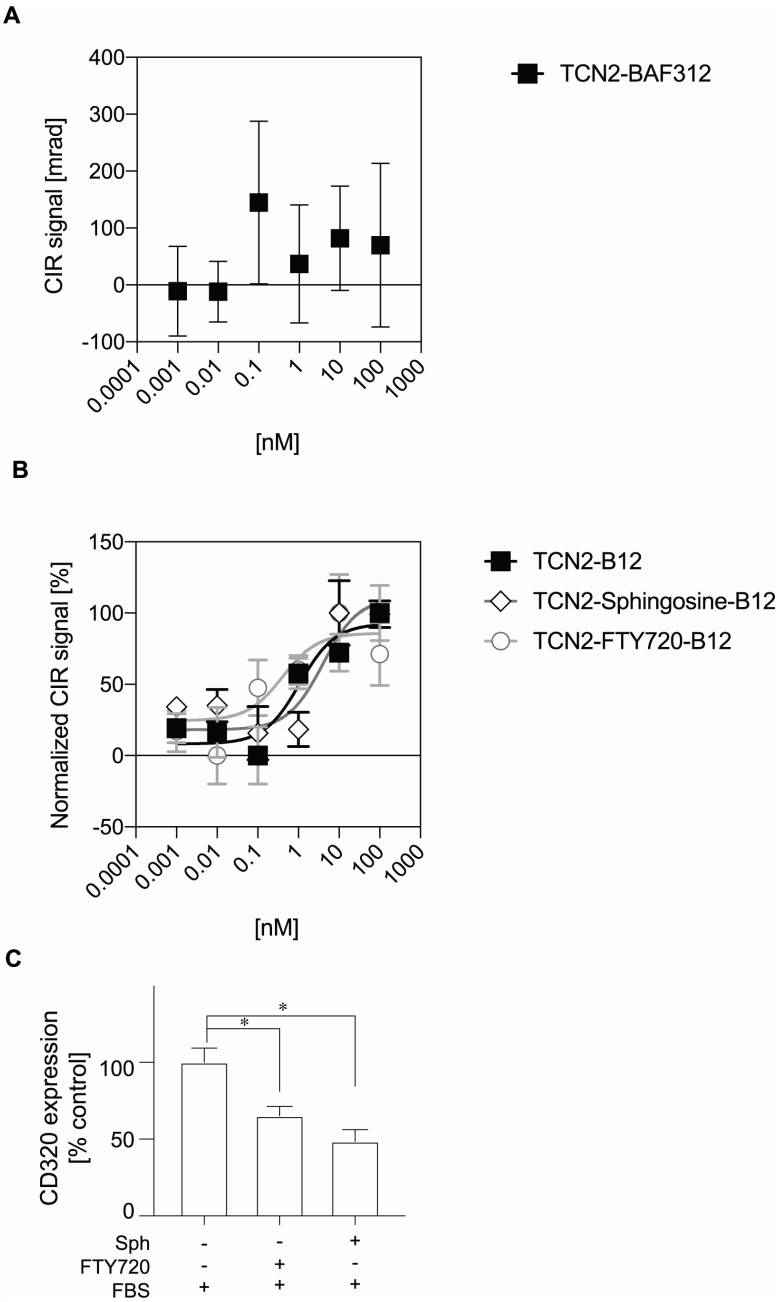


Fig. S4. Compensated interferometric reader (CIR)-based binding assay and CD320 internalization. A, No clear binding signals between TCN2 vs. BAF312 (siponimod). **B**, Specific binding curves between TCN2-B_{12} (Kd = 5.98 ± 8.96 nM) vs. FTY720 (Kd = 5.62 ± 5.24 nM) and sphingosine (Kd = 0.33 ± 0.33 nM). Compensated Interferometric Reader (CIR) signals are plotted against concentrations of binding partners, normalized, and fitted by nonlinear regression using the Michaelis-Menten equation (mean ± SEM, n = 4-5, p = 0.34 by One-way ANOVA). **C**, CD320 internalization in HASTR/ci37 cells. Cells were stimulated with 1 μM FTY720 or 1 μM Sph in the presence of FBS. * $p < 0.05$, one-way ANOVA with Bonferroni's multiple

comparisons test.

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