SUPPLEMENTARY FIGURES

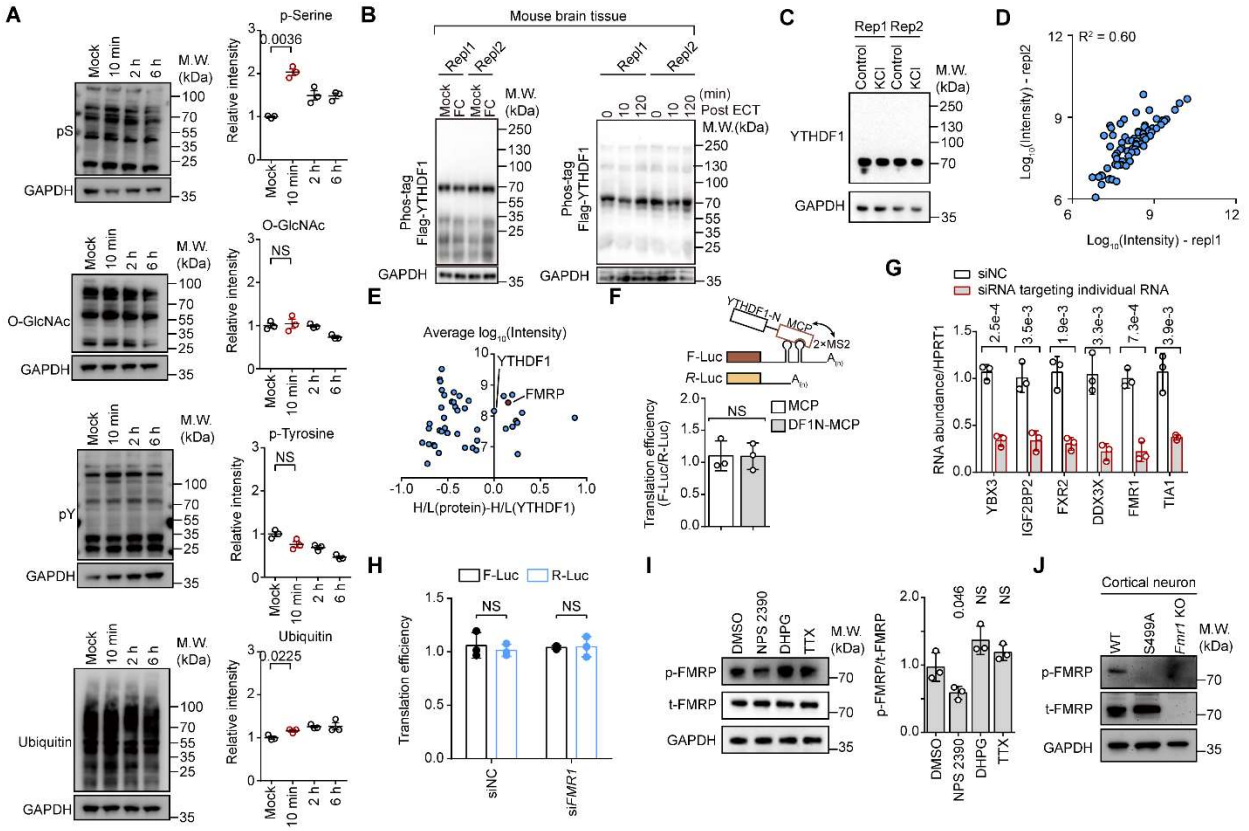

Figure S1 FMRP is phosphorylated at S499 upon neuronal depolarization. Related to Figure 1.

(A) Representative western blots of global post-translational modifications upon neuronal depolarization (*n* = 3 each condition). Western blots are quantified by ImageJ. Relative intensities of different proteins are quantified by normalizing to GAPDH.

(B) Phos-tag gels assaying YTHDF1 phosphorylation in the mouse brain after fear conditioning (FC) or electroconvulsive treatment (ECT).

(C) Phos-tag gel analyzing YTHDF1 in cultured mouse neurons after KCl depolarization.

(D) Scatter plot characterizing the reproducibility between the two replicates for identifying YTHDF1 protein partners using mass spectrometry.

(E) Scatter plot showing the intensity of individual protein identified in fractions co-immunoprecipitated with YTHDF1.

(F) Schematics showing the experimental setup for the reporter assay to study YTHDF1-mediated translation in HEK293T cells transfected with siControl siRNA. The N terminus of YTHDF1 was tethered to the reporter firefly luciferase RNA by MCP-MS2 interactions. Bar plot showing that YTHDF1 tethering in WT HEK293T cells does not alter target RNA translation.

(G) qPCR results validating individual knockdown of candidate proteins that negatively regulates m^6A-mediated translation. Individual RNA level was normalized to *HPRT1*.

(H) Bar plot showing that *FMR1* knockdown did not alter the translation efficiency of untethered reporter firefly luciferase or renilla luciferase coding sequences.

(I) Western blots showing response of FMRP phosphorylation to different small molecules in cultured neurons. DMSO was used as a control and cellular extracts were prepared at 5 minutes after application of drugs. FMRP phosphorylation levels were obtained by normalizing band intensity of p-FMRP to band intensity of t-FMRP. The final concentrations of individual small molecules were determined based on their reported IC_{50} values against their physiological targets (NPS 2390: 82 nM, DHPG, 7 μM, and TTX, 2 nM).

(J) Western blotting of mouse hippocampal neurons validating knockout of *Fmr1* gene.

Data are mean ± s.e.m. (A), (F), (G), (H) and (I), statistical analysis was performed using unpaired two-tailed *t*-test with Welch's correction. Exact *P* values are indicated, and NS denotes *P* values > 0.05.

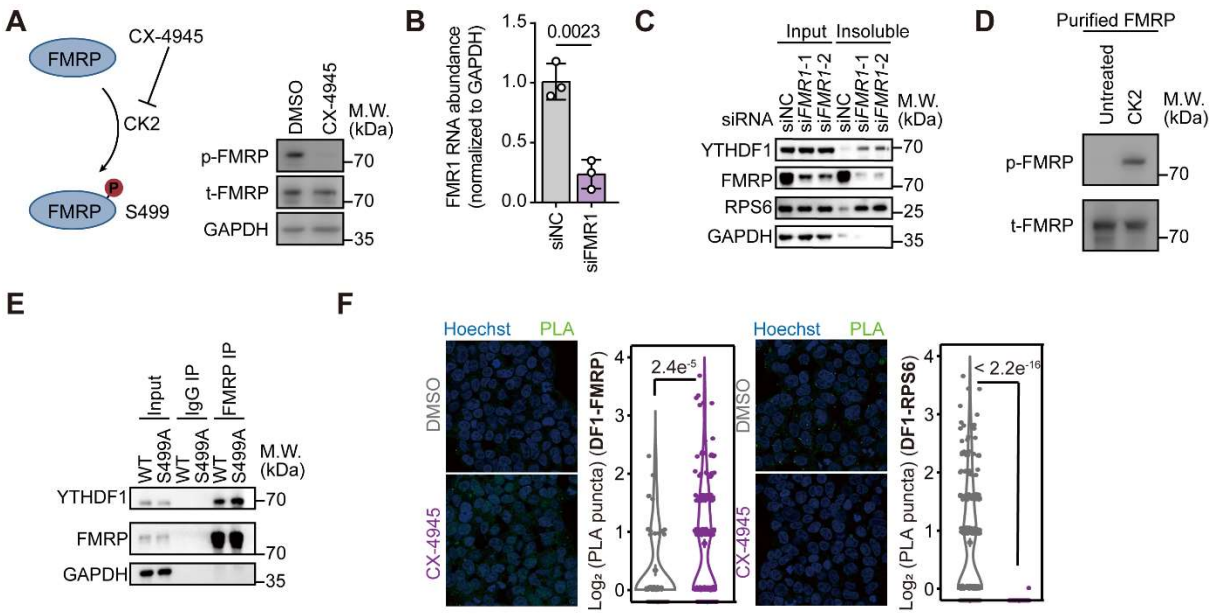

Figure S2 FMRP and its phosphorylation regulates YTHDF1 condensing. Related to Figure 2.

(A) Left: illustration of phosphorylation inhibitor treatment. Right: western blot of FMRP S499 phosphorylation in HeLa cells treated by CX-4945.

(B) qPCR results showing knockdown efficiency of *FMR1* RNA in HeLa cells.

(C) Western blots of insoluble fractions isolated from HeLa lysates. YTHDF1 and RPS6 were both enriched after *FMR1* knockdown.

(D) Western blots characterizing phosphorylated recombinant FMRP with CK2 *in vitro*. Unphosphorylated FMRP was recombinantly purified from BL21(DE3). Phosphorylation reaction was performed with recombinant casein kinase 2 enzyme.

(E) Western blots showing YTHDF1-FMRP associations. Immunoprecipitations were performed with FMRP antibody in HeLa cells overexpressing WT or phosphor-null mutant (S499A) of FMRP.

(F) Proximity ligation assay quantifying interactions between YTHDF1/FMRP or YTHDF1/RPS6.

Data are mean ± s.e.m. (B), statistical analysis was performed using unpaired two-tailed *t*-test with Welch's correction. (F), statistical analysis was performed with Wilcoxon rank sum test. Exact *P* values are indicated, and NS denotes *P* values > 0.05.

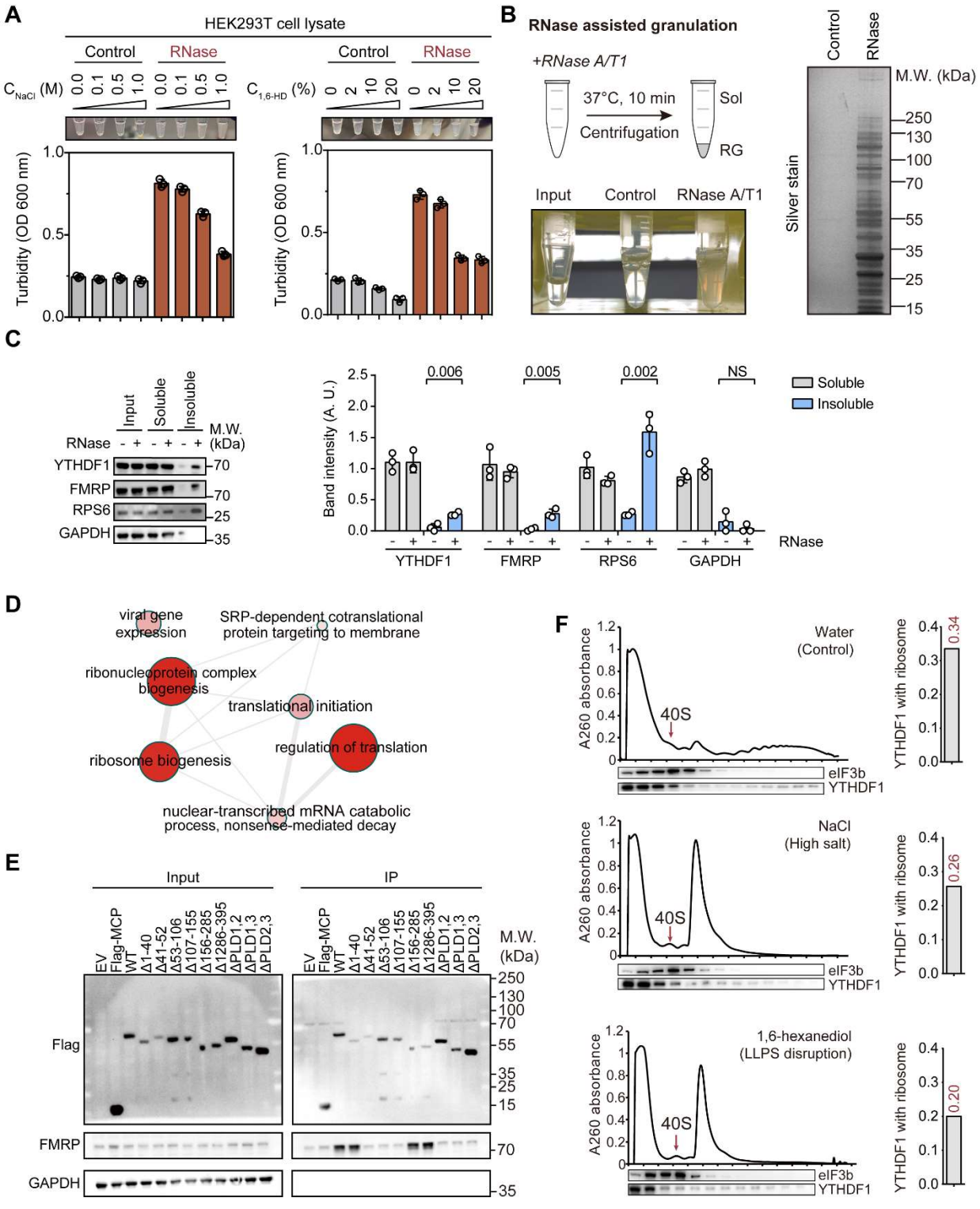

Figure S3 RNase treatment precipitates condensates containing YTHDF1 and ribosomal proteins. Related to Figure 3.

(A) Top: representative images of the turbidity of HEK293T lysates treated by RNase and high salt (NaCl) or 1,6-hexanediol (1,6-HD). Bottom: turbidity measured by OD 600 nm from nanodrop.

(B) Analysis of proteins precipitated by RNase treatment. Top left: illustration of experimental design. Bottom left: representative images of the RNase-induced granulation formation. Right: silver stain of proteins present in the condensed phase in response to RNase treatment.

(C) Distribution of YTHDF1, FMRP and RPS6 in the condensed phase in response to RNase treatment. Quantifications of western blots were performed by normalizing intensities of each band to that of “Input RNase –” lane of each protein assayed.

(D) Gene ontology terms enriched by condensation-dependent YTHDF1 protein partners. GO enrichment was done with ClusterProfiler algorithm built in R.

(E) Western blots showing expression of different YTHDF1 mutants in HeLa cells and their interactions with FMRP.

(F) Relative distribution of *YTHDF1* in polysome fractions. *YTHDF1* percentage in the ribosomal fractions was quantified by normalizing intensity of YTHDF1 in 40S, 60S and 80S ribosome to total intensity. 1,6-hexanediol was added at 2% (w/v) concentration.

Data are mean ± s.e.m. (C), statistical analysis was performed using unpaired two-tailed *t*-test with Welch’s correction. Exact *P* values are indicated, and NS denotes *P* values > 0.05.

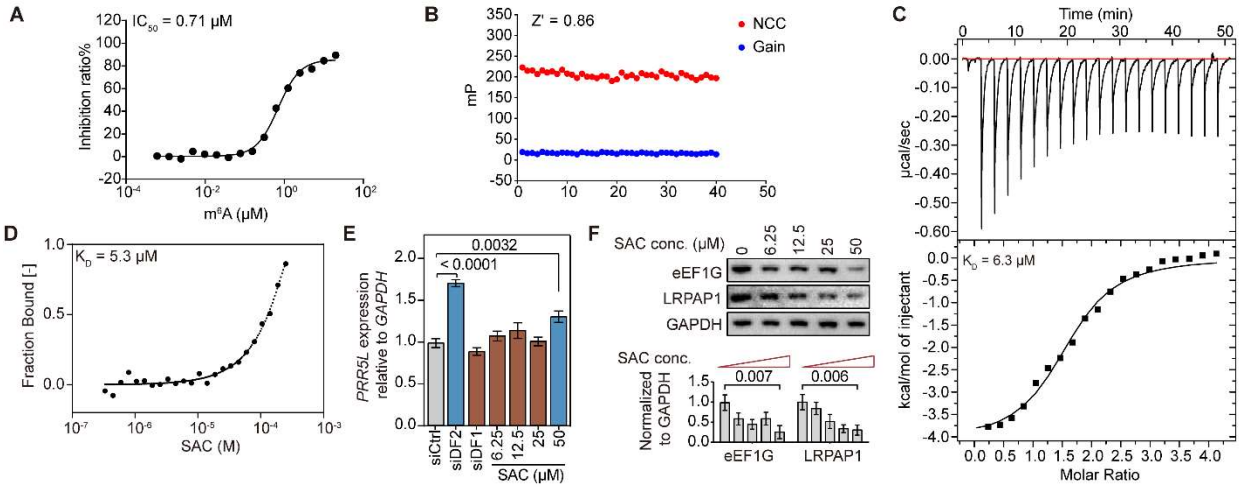

Figure S4 SAC selectively inhibits YTHDF1 at low concentrations. Related to Figure 4.

(A) IC_{50} of m^6A-containing mRNA (m^6A) to compete with YTHDF1-target binding as measured by AlphaScreen experiments. Different concentrations of unlabeled m^6A-containing mRNA were added to compete with fluorescence molecule for YTHDF1 as indicated.

(B) Z' scores to characterize the stability of small molecule inhibitor screening assay. The experiment conditions (NCC and Gain) were repeated 40 times as indicated. Z' was calculated based on the values of *mP*.

(C) ITC analysis between SAC and YTHDF1 at 25 °C. 1 mM SAC was titrated into 50 μM YTHDF1.

(D) The binding curve of SAC and YTHDF1 obtained via MST assay.

(E) RNA levels of YTHDF2 target (*PRR5L*) upon gradient SAC treatments by a concentration gradient. *PRR5L* expressions were quantified by normalizing RNA levels to *GAPDH*.

(F) Top: western blots showing the protein levels of YTHDF1 targets (eEF1G and LRPAP1) upon SAC treatments by a concentration gradient. Bottom: protein levels relative to GAPDH quantified by ImageJ (n = 3).

Data are mean ± s.e.m. Statistical analysis was performed using unpaired two-tailed *t*-test with Welch's correction. Exact *P* values are indicated, and NS denotes *P* values > 0.05.

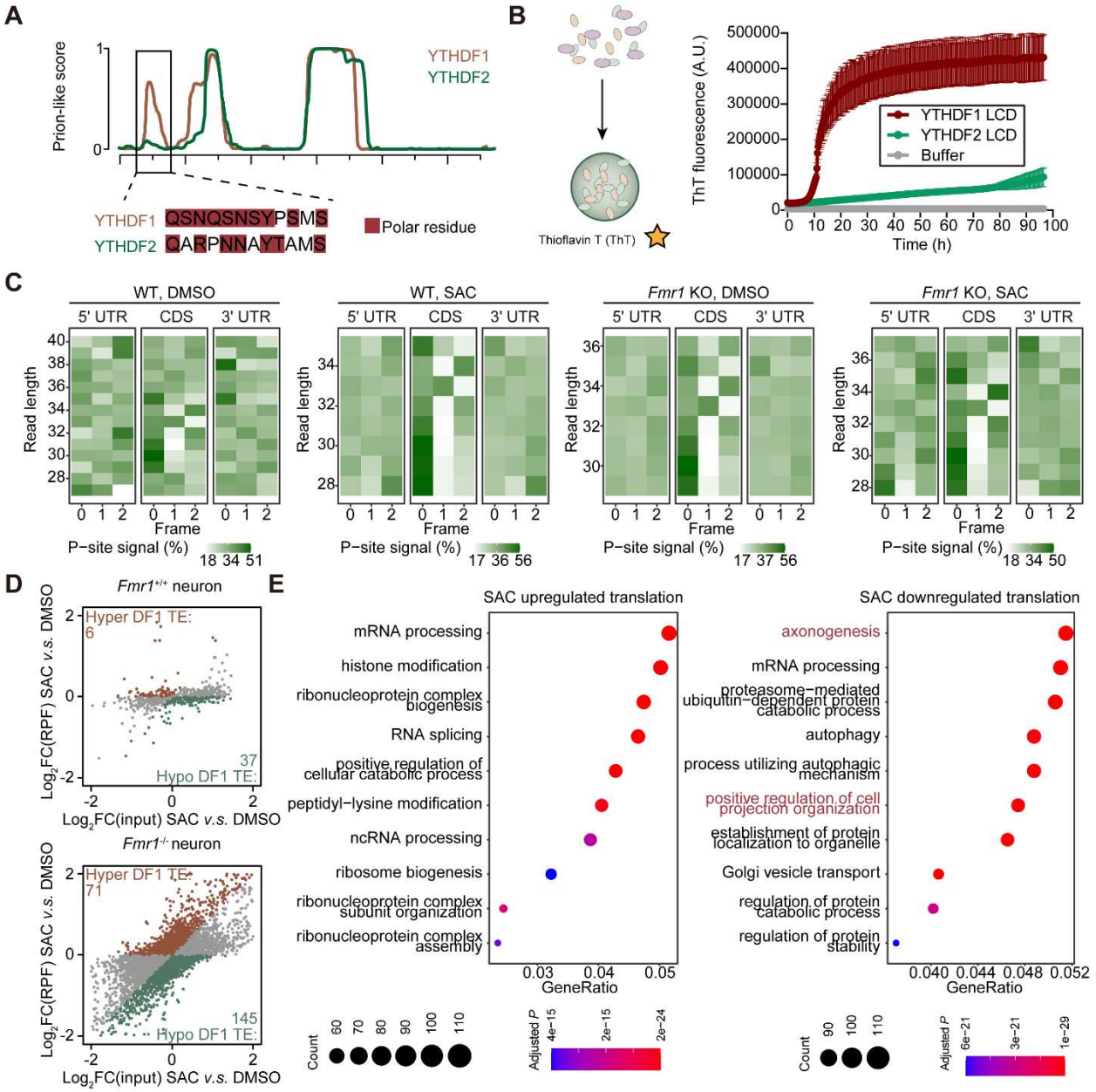

Figure S5 YTHDF1 and YTHDF2 differ in their N-terminal intrinsically disordered domain sequence and exhibit different fiber formation behaviors. Related to Figure 5.

(A) Prion-like score of YTHDF1 and YTHDF2 predicted by PLAAC.

(B) Thioflavin T (ThT) assay of YTHDF1 and YTHDF2 low-complexity domains. Freshly purified proteins were incubated for 100 hours in native buffer.

(C) Trinucleotide periodicity of P-sites identified with ribosome-protected fragments in individual samples showing data quality of riboLace-seq.

(D) Scatter plots showing both ribosome-protected fragments (RPF) and input RNA level (input) obtained by riboLace-seq. Data for WT and *Fmr1* KO neurons were shown.

(E) Dot plots showing the gene ontology terms enriched for hyper-translated (left) or hypo-translated (right) genes upon SAC treatment. Top biological process terms enriched were shown.

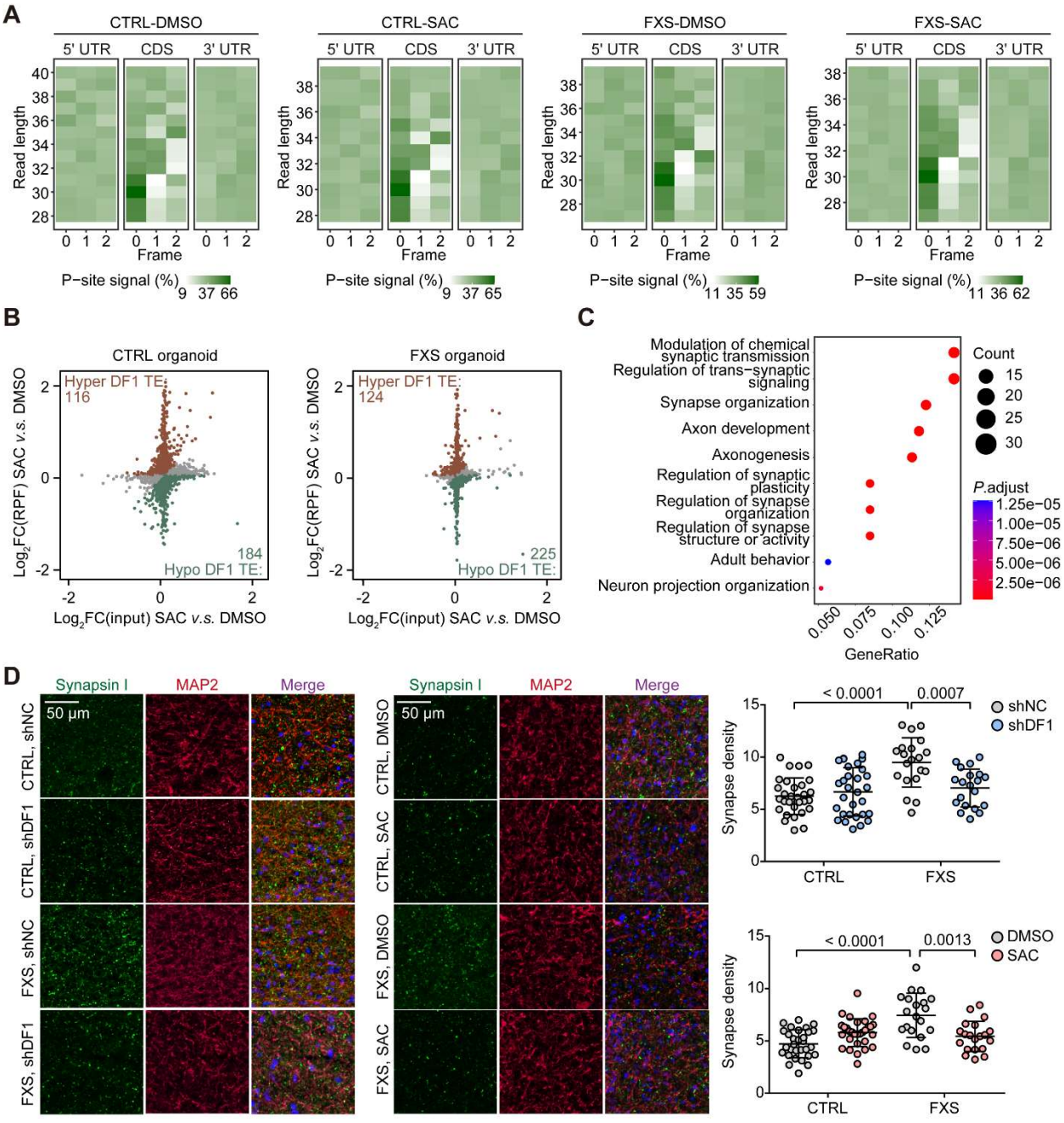

Figure S6 RiboLace measures translation events in organoid models. Related to Figure 6.

(A) Trinucleotide periodicity of P-sites identified with ribosome-protected fragments in individual samples indicating the good data quality of RiboLace-seq.

(B) Scatter plots showing the changes in RNA translation in forebrain organoids upon SAC treatment.

(C) Gene ontology terms enriched by hypo-translated genes in FXS organoids upon SAC treatment.

(D) Representative images and quantification showing the synaptic density in FXS or control (CTRL) organoids. Knockdown of *YTHDF1* or SAC treatment was performed. The synaptic density was calculated the number of SYNAPSIN I positive puncta per 100 um^2 MAP2 (microtubule associated protein 2) positive area. Data are presented as mean ±s.d (n = 10 sections from at least from 2-3 organoids each condition). Statistical analysis was performed using two-way ANOVA. Scale bars: 50 μm.