SUPPLEMENTARY MATERIAL

The m^{6}A reader YTHDF1 regulates axon guidance through translational control of Robo3.1 expression

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Supplementary Figure S1. Labeling of commissural axons with GFP. (**A**) Cross section of spinal cord of *Atoh1-Cre$^{+/-}$;Rosa26$^{mT/mG}$* embryo at E11.5 showed GFP labelling in dorsal commissural neurons (DCN) and commissural axons (CA). (**B**) Co-immunostaining of GFP (green) and Robo3.1 (red) in cross section of ventral spinal cord of *Atoh1-Cre$^{+/-}$;Rosa26$^{mT/mG}$* embryo at E11.5. Robo3.1 was expressed in pre-crossing and crossing commissural axons (yellow arrows) and was absent from post-crossing axons (green arrowheads). (**C**) Immunostaining of E11.5 spinal cord sections with two Robo3.1 antibodies (gt, goat polyclonal; rt, rabbit polyclonal from Marc Tessier-Lavigne lab) showing identical patterns. (**D** and **E**) RT-qPCR demonstrated that application of FP-CM, CHX or MG-132 to cultured DCN explants did not change *Robo3.1* mRNA levels. Quantification of RT-qPCR is represented as dot plots (*n* = 3 replicates): ns, not significant; for D, *P* = 0.23 (Ctrl *vs* FP-CM), *P* = 0.13 (Ctrl *vs* CHX), by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test; for E, *P* = 0.34 (Vehicle *vs* MG-132), by unpaired Student's *t* test. Scale bars, 100 μm (A) and 50 μm (B and C).

Supplementary Figure S2. *Robo3.1* mRNA is predicted to be modified by m^{6}A and loss of function of m^{6}A writer METTL3 does not change *Robo3.1* mRNA levels. (**A**) All predicted m^{6}A sites in full length of *Robo3.1* mRNA with SRAMP. High Confidence m^{6}A sites were mutated in this work. (**B**) *Robo3.1* mRNA was not changed in neurons treated with *shMettl3*, compared with *shCtrl*. Quantification of RT-qPCR is represented as dot plots (*n* = 3 replicates): ns, not significant (*P* = 0.25); by unpaired Student's *t* test. (**C**) Similar YTHDF1 proteins levels were detected in input samples, and also RIP using YTHDF1 antibody

pulled down similar YTHDF1 protein from COS-7 cells co-expressing YTHDF1 and *Robo3.1* with m^6A sites mutated (MT^{m6A}) compared with *WT Robo3.1*.

Supplementary Figure S3. YTHDF1 does not change *Robo3.1* mRNA levels and YTHDF2 does not regulate translation of *Robo3.1*. (**A** and **D**) RT-qPCR analysis showed that YTHDF1 could not change *Robo3.1* mRNA levels in cells co-expressing Robo3.1 and YTHDF1 under different conditions as indicated. Quantification of RT-qPCR is represented as dot plots (n = 3 replicates): ns, not significant; for A, P = 0.12 ("*Robo3.1-WT + IRES-eGFP*" *vs* "*Robo3.1-WT + Ythdf1-IRES-eGFP*"), P = 0.13 ("*Robo3.1-WT + IRES-eGFP*" *vs* "*Robo3.1-MT^{m6A} + Ythdf1-IRES-eGFP*"), P = 0.95 ("*Robo3.1-WT + Ythdf1-IRES-eGFP*" *vs* "*Robo3.1-MT^{m6A} + Ythdf1-IRES-eGFP*"); for D, P = 0.66 ("HA-Robo3.1" *vs* "HA-Robo3.1 + CHX"), P = 0.71 ("HA-Robo3.1" *vs* "HA-Robo3.1 + YTHDF1"), P = 0.37 ("HA-Robo3.1 + YTHDF1" *vs* "HA-Robo3.1 + YTHDF1 + CHX"); by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. (**B**) YTHDF2 could not change Robo3.1 protein level. Co-expression of WT Robo3.1 with YTHDF2 in COS-7 cells did not lead to significant changes of Robo3.1 protein level by IF, compared with the control. Scale bar, 25 μm. (**C**) Quantification of relative Robo3.1 IF to eGFP in (B). Data of IF quantification (C) are represented as box and whisker plots: "*Robo3.1-WT + IRES-eGFP*" (n = 24 cells) *vs* "*Robo3.1-WT + Ythdf2-IRES-eGFP*" (n = 36 cells), ns, not significant (P = 0.09); by unpaired Student's t test.

Supplementary Figure S4. YTHDF1 expression in DSC drops from pre-crossing to post-crossing stages. (**A** and **B**) Knockdown or overexpression of YTHDF1 did not change *Robo3.1* mRNA levels in commissural neurons. RT-qPCR analysis showed that *Robo3.1* mRNA levels were not affected after YTHDF1 was knocked down (A) or overexpressed (B) in commissural neurons. (**C**) YTHDF1 protein levels in DCN were decreasing from embryonic stage E10.5 to E12.5. Total Protein was extracted from mouse embryonic dorsal spinal cord of different stages with RIPA buffer, followed by anti YTHDF1 Western Blotting. As

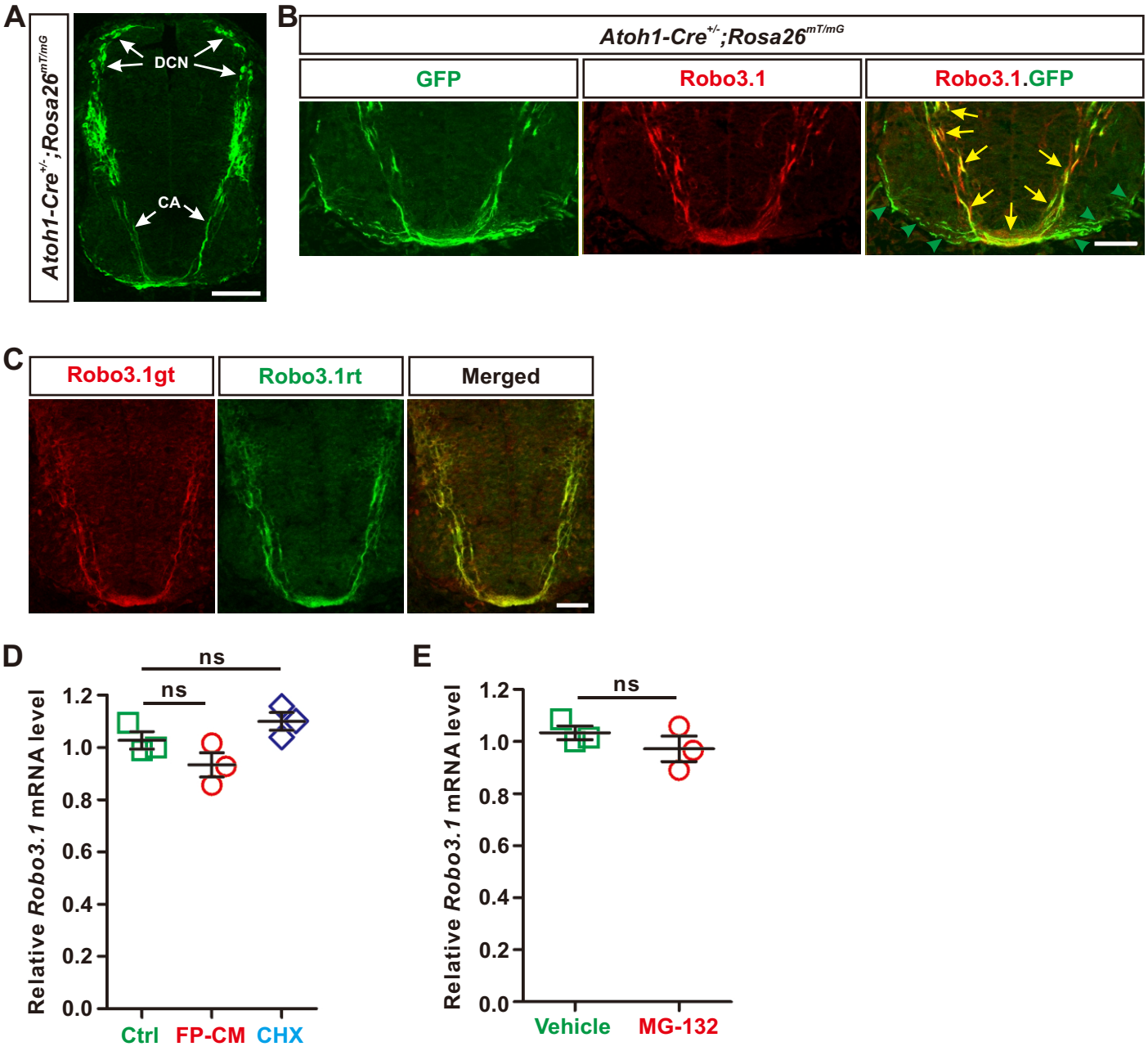
shown, YTHDF1 protein was continuously decreasing from E10.5 to E11.5, and to E12.5. (**D**) Quantification of WB results in (C). Quantification of RT-qPCR and WB is represented as dot plots (*n* = 3 replicates): ns, not significant; for A, *P* = 0.99 (*shYThdf1-2 vs shCtrl*), *P* = 0.43 (*shYThdf1-3 vs shCtrl*), by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test; for B, *P* = 0.62, by unpaired Student's *t* test; for D, ****P* = 3.65E-5 (E10.5 *vs* E11.5), ***P* = 0.0016 (E11.5 *vs* E12.5), by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

Supplementary Figure S5. *Ythdf1* cKO does not change *Robo3.1* mRNA levels, patterning of spinal cord, or commissural neuron development. (**A**) RT-qPCR analysis showing that *Robo3.1* mRNA level was not altered in *Ythdf1* cKO embryos. Quantification of RT-qPCR is represented as dot plots (*n* = 3 replicates): ns, not significant; *P* = 0.09; by unpaired Student's *t* test. (**B**) Isl1/2 immunostaining in E11.5 spinal cord indicated that *Ythdf1* cKO does not disturb neural patterning in neural tube development. (**C**-**E**) Quantification of Isl1/2$^+$ spinal neuron subtypes in (B). (**F**-**I**) Development of dI1 commissural neurons marked by Lhx9 (F-representative images, G-quantification) and Lhx2 (H-representative images, I-quantification) is not affected in *Ythdf1* cKO. All data are mean ± s.e.m. and represented as box and whisker plots: *Ythdf1*$^{fl/fl}$ (*n* = 10 sections in C-E; *n* = 15 sections in G; *n* = 12 sections in I) *vs Atoh1-Cre*$^{+/-}$*;Ythdf1*$^{fl/fl}$ (*n* = 11 sections in C-E; *n* = 15 sections in G; *n* = 9 sections in I); ns, not significant (*P* = 0.65 for Isl1/2$^+$ motor neurons in C; *P* = 0.20 for Isl1/2$^+$ preganglionic column neurons in D; *P* = 0.97 for Isl1/2$^+$ dI3 neurons in E; *P* = 0.47 for Lhx9$^+$ neurons in G; *P* = 0.13 for Lhx2$^+$ neurons in I); by unpaired Student's *t* test. Scale bars, 100 μm (B, F and H).

Supplementary Figure S6. m^{6}A modification level changes, but not another m^{6}A reader YTHDF3, contribute to translational regulation of *Robo3.1*. (**A**) The m^{6}A levels of *Robo3.1* mRNA were decreased after stage E10.5. Total RNA was extracted from E10.5 (8 embryos), E11.5 (8 embryos) and E12.5 (7

embryos) mouse embryonic spinal cord, followed by anti m^6A IP. RT-qPCR analysis of RNA elutes showed dramatic decreases of m^6A levels from E10.5, E11.5 to E12.5. Quantification of RT-qPCR is represented as dot plots (*n* = 3 replicates): ****P = 1.52E-5 (E10.5 *vs* E11.5), ****P = 3.52E-6 (E10.5 *vs* E12.5), by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. (**B**-**E**) Loss of function of YTHDF3 did not significantly change *Robo3.1* mRNA or proteins levels. (**B**) Western Blotting confirmed that *shYthdf3* could efficiently knock down YTHDF3 in DSC neurons. (**C**) Robo3.1 Immunofluorescence in commissural axons showed no change after *shYthdf3* infection of commissural neurons which are marked by eGFP. Scale bar, 10 μm. (**D**) Quantification of Robo3.1 IF in (C). All data are mean ± s.e.m. and represented as box and whisker plots: ns, not significant; P = 0.053; by unpaired Student's *t* test. (**E**) RT-qPCR analysis showed that knockdown of YTHDF3 in commissural neurons did not change *Robo3.1* mRNA levels. Quantification of RT-qPCR is represented as dot plots (*n* = 3 replicates): ns, not significant; P = 0.70; by unpaired Student's *t* test.

Supplementary Figure S1



Supplementary Figure S2

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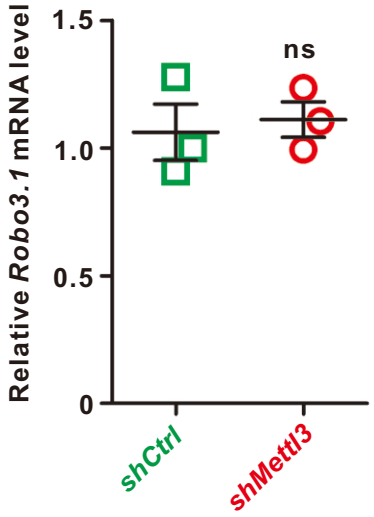
AUG: Start codon
UGA: Stop codon
GGACA: predicted High Confidence m^{6}A site, which are mutated in this work
GAACU: predicted Moderate or Low confidence m^{6}A site

B



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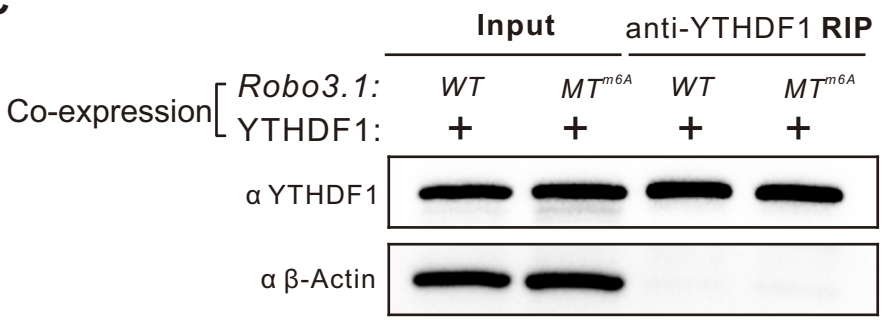
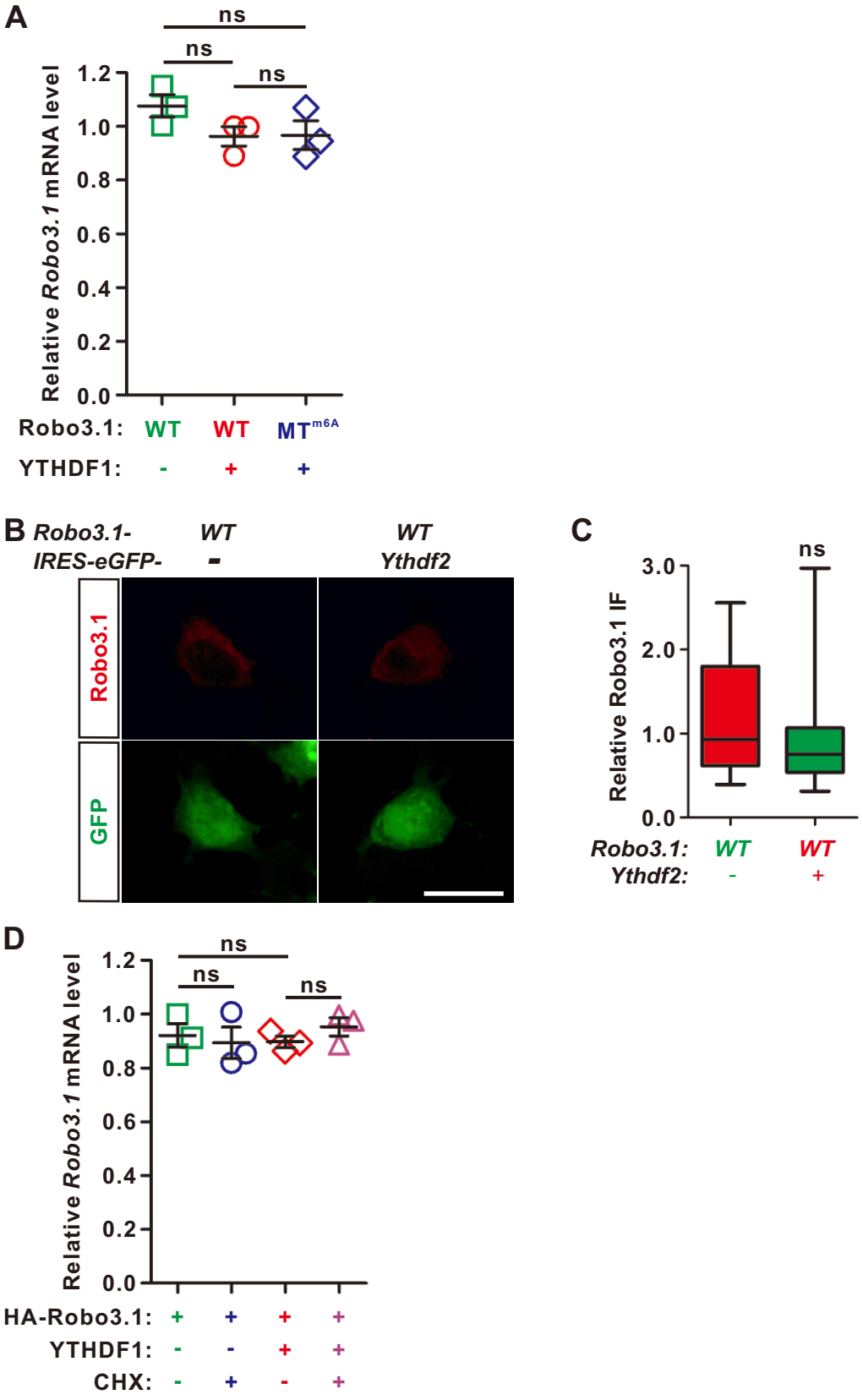
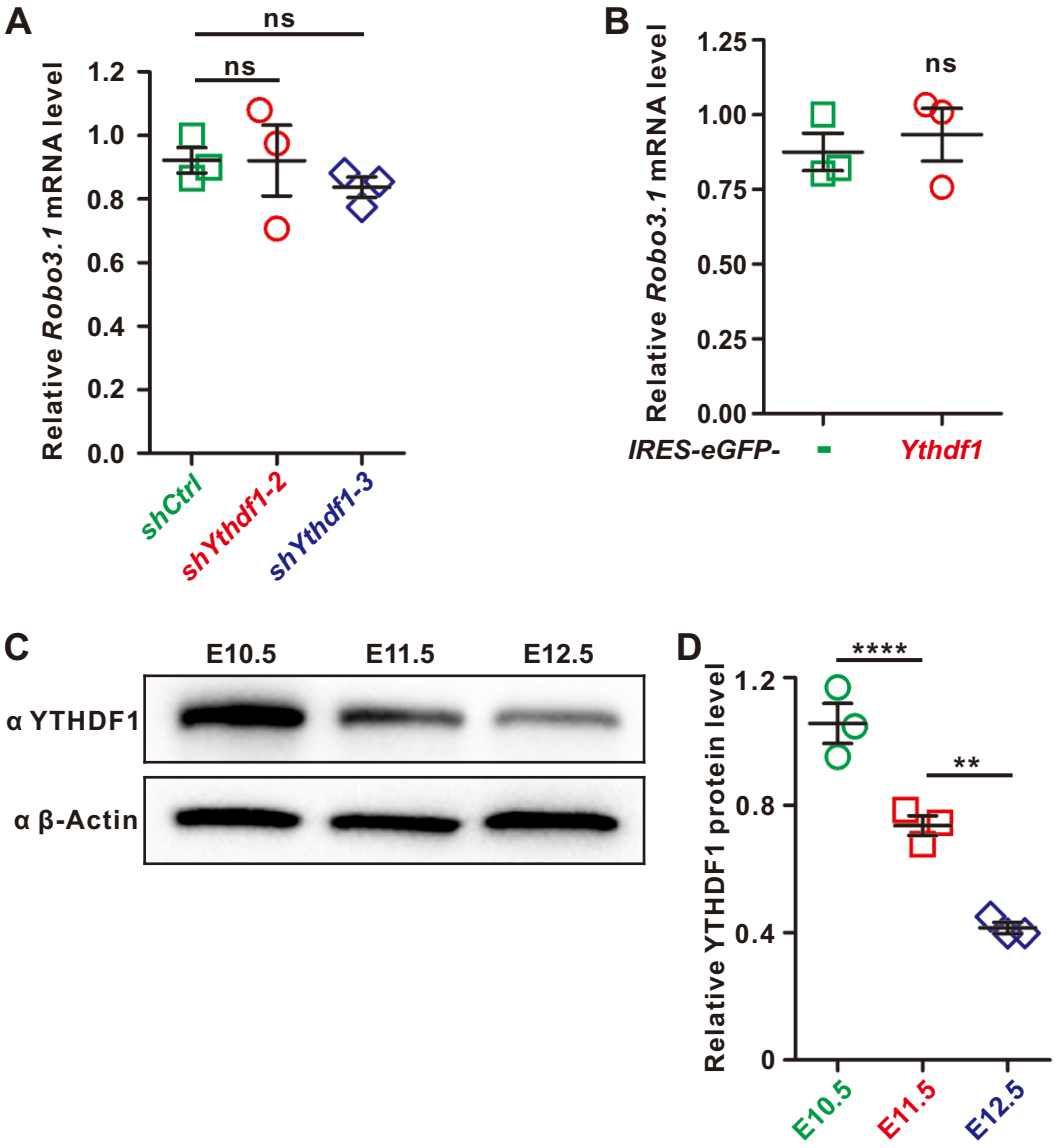


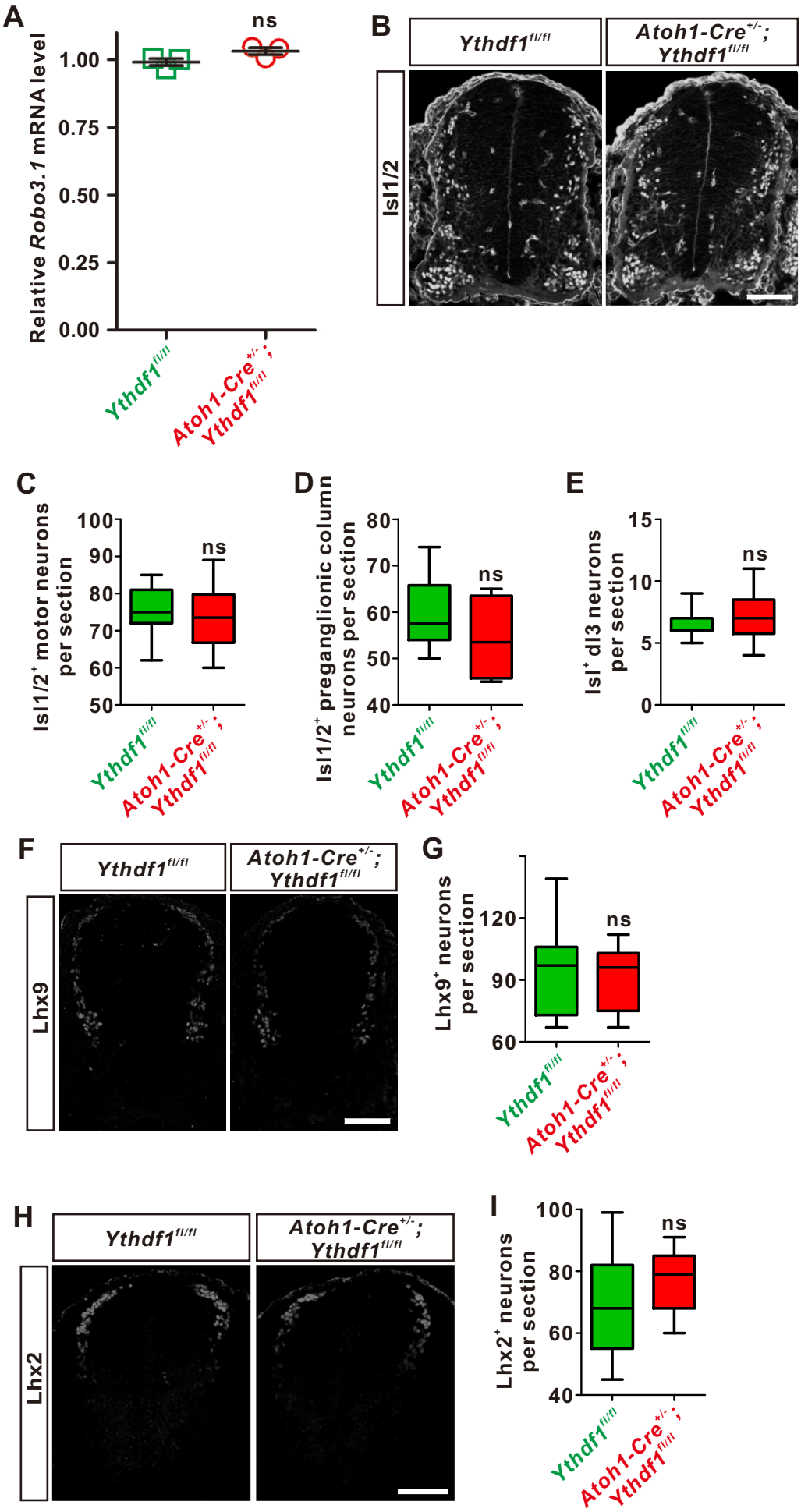
Supplementary Figure S3



Supplementary Figure S4



Supplementary Figure S5



Supplementary Figure S6