RESEARCH ARTICLE

Figures and figure supplements

Aberrant cortical development is driven by impaired cell cycle and translational control in a *DDX3X* syndrome model

Mariah L Hoye *et al*

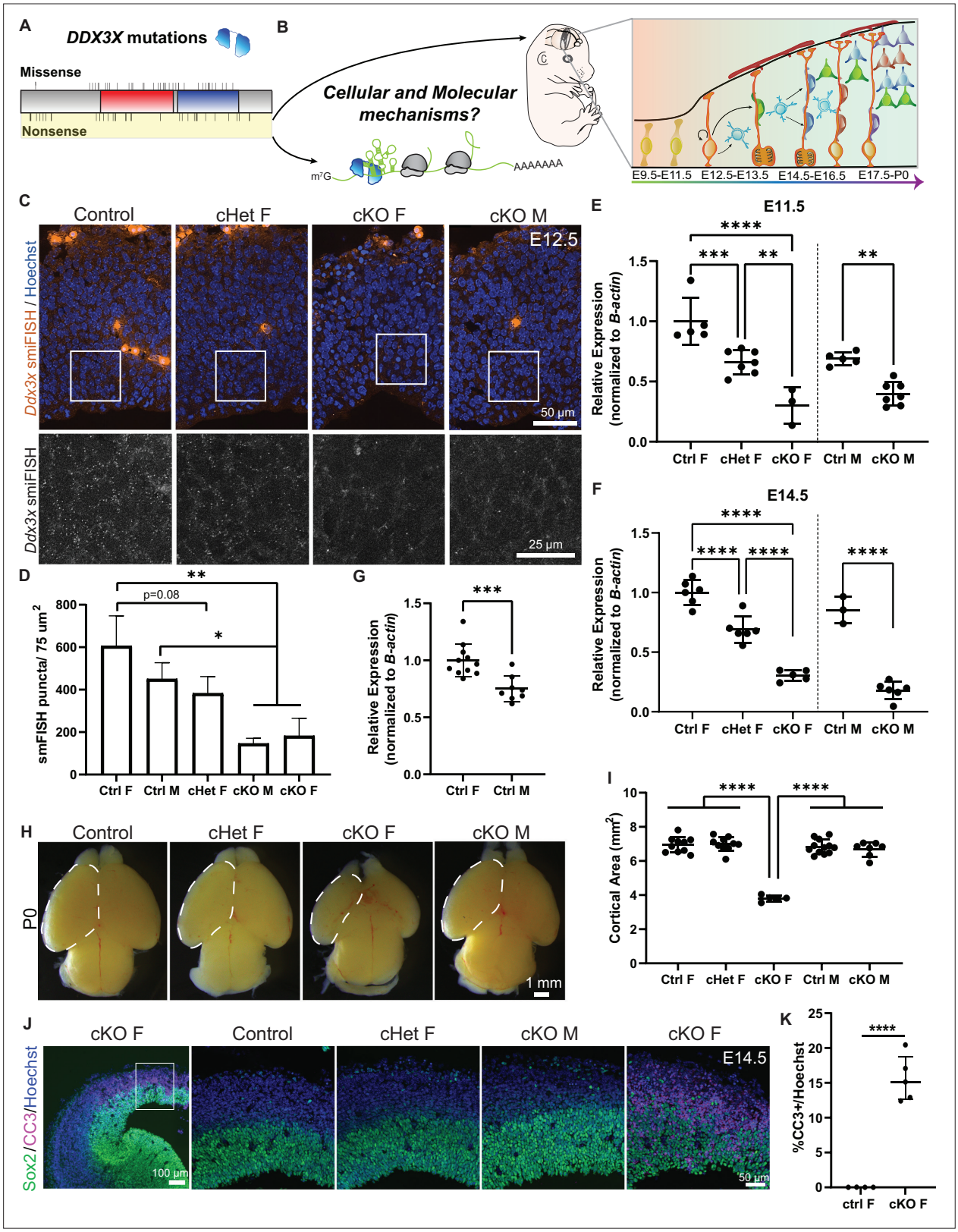

Figure 1. Conditional knockout of *Ddx3x* in neural progenitors using *Emx1*-Cre leads to microcephaly in female mice. (**A**) Schematic of DDX3X protein with human missense and nonsense mutations noted, along with helicase/RNA binding domains (red, blue). Nonsense mutations, highlighted in yellow, are predicted to act in a LoF manner. (**B**) (Left) DDX3X protein bound to an mRNA undergoing translation. (Right) Mouse embryo and corticogenesis showing neuroepithelial cells (light green), radial glial cells (RGCs, orange), intermediate progenitors (IPs, light blue), and neurons

Figure 1 continued on next page

Figure 1 continued

(multi-colored); ***Figure 1B*** adapted from Figure 1A and B from ***Hoye and Silver, 2021***. This study asks how does *Ddx3x* LoF impair mouse embryonic cortical development at a cellular and molecular level? (**C**) Representative sections of smFISH for *Ddx3x* in control, cHet female, and cKO male and female E12.5 cortices. (**D**) Quantification of *Ddx3x* smFISH signal in respective genotypes at E12.5. n=2–3 embryos/condition (**E, F**) Validation of *Ddx3x* mRNA knockdown in Tdtomato + cells from female (**F**) (control, cHet, cKO) and male (**M**) (control, cKO) brains sorted via FACS at E11.5 (**E**) and E14.5 (**F**). n=3–7 embryos/condition. (**G**) Quantification of *Ddx3x* levels in Tdtomato + cells from control female and male brains. n=8–10 embryos/condition. (**H**) Representative whole mount images of control, cHet female, and cKO male and female brains at P0. (**I**) Quantification of cortical area at P0. n=5–12 embryos/condition. (**J**) Representative sections of E14.5 brains stained with Sox2 (green), CC3 (magenta) and Hoechst (blue) showing low-magnification on left panel, and high magnification on 4 panels to the right. (**K**) Quantification of CC3 + cells in E14.5 control and cKO female cortices. n=4–5 embryos/condition. Scale bars, indicated. Error bars, S.D. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. One-way ANOVA with Tukey's (**D, E, F, I**), Student's unpaired, two-tailed t-test (**G, K**).

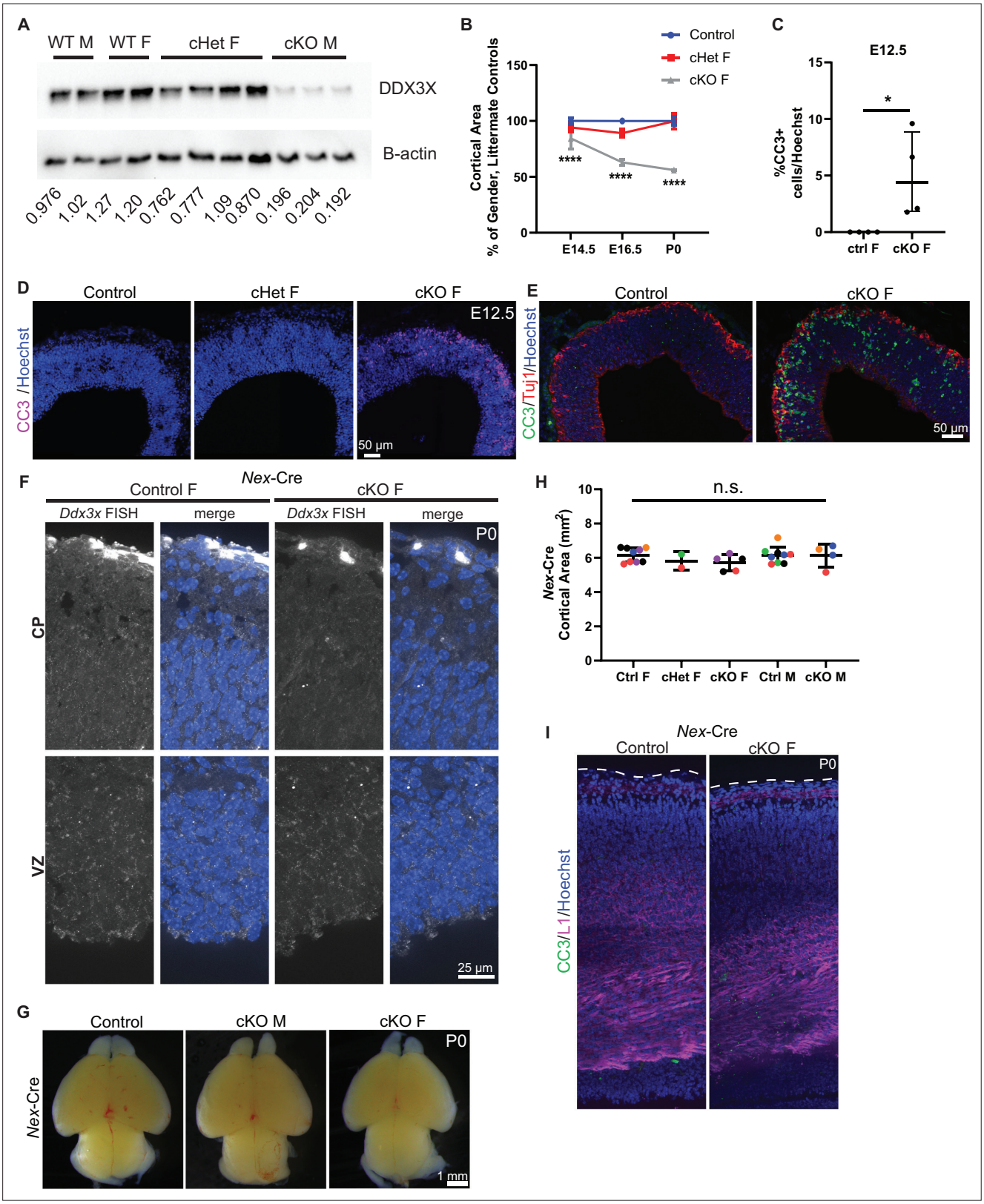

Figure 1—figure supplement 1. *Ddx3x* loss from neural progenitors, but not neurons leads to microcephaly and apoptosis. (**A**) Representative western blot of indicated genotypes probed for DDX3X (top) and β-actin loading control (bottom). Densitometric quantification of bands is shown below each lane. These data also indicate this antibody is specific to DDX3X and does not cross react with DDX3Y. n=2–4 embryos/condition. (**B**) Temporal quantification of cortical area in control, cHet F, and cKO F at E14.5, E16.5 and P0. n=3–12 embryos/condition. (**C**) Quantification of CC3 + cells in

Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

control and cKO F at E12.5. n=4 embryos/condition. (**D**) Representative coronal sections from control, cHet F, and cKO F at E12.5 immunostained with CC3 (magenta) and Hoechst. (**E**) Representative coronal sections from control and cKO F at E12.5 immunostained with CC3 (green), Tuj1 (red) and Hoechst. (**F**) Representative coronal sections of smiFISH for *Ddx3x* mRNA in *Nex*-Cre control and cKO female cortices at P0 showing the ventricular zone (VZ) and cortical plate (CP). (**G**) Representative whole mount images of *Nex*-Cre control and cKO male and female brains at P0. (**H**) Quantification of *Nex*-Cre cortical area at P0. n=2–9 embryos/condition. (**I**) Representative coronal sections from *Nex*-Cre control and cKO F at P0 immunostained with CC3 (green), L1 (magenta) and Hoechst. One-way ANOVA with Tukey's (**B, H**), Student's unpaired, two-tailed t-test (**C**).

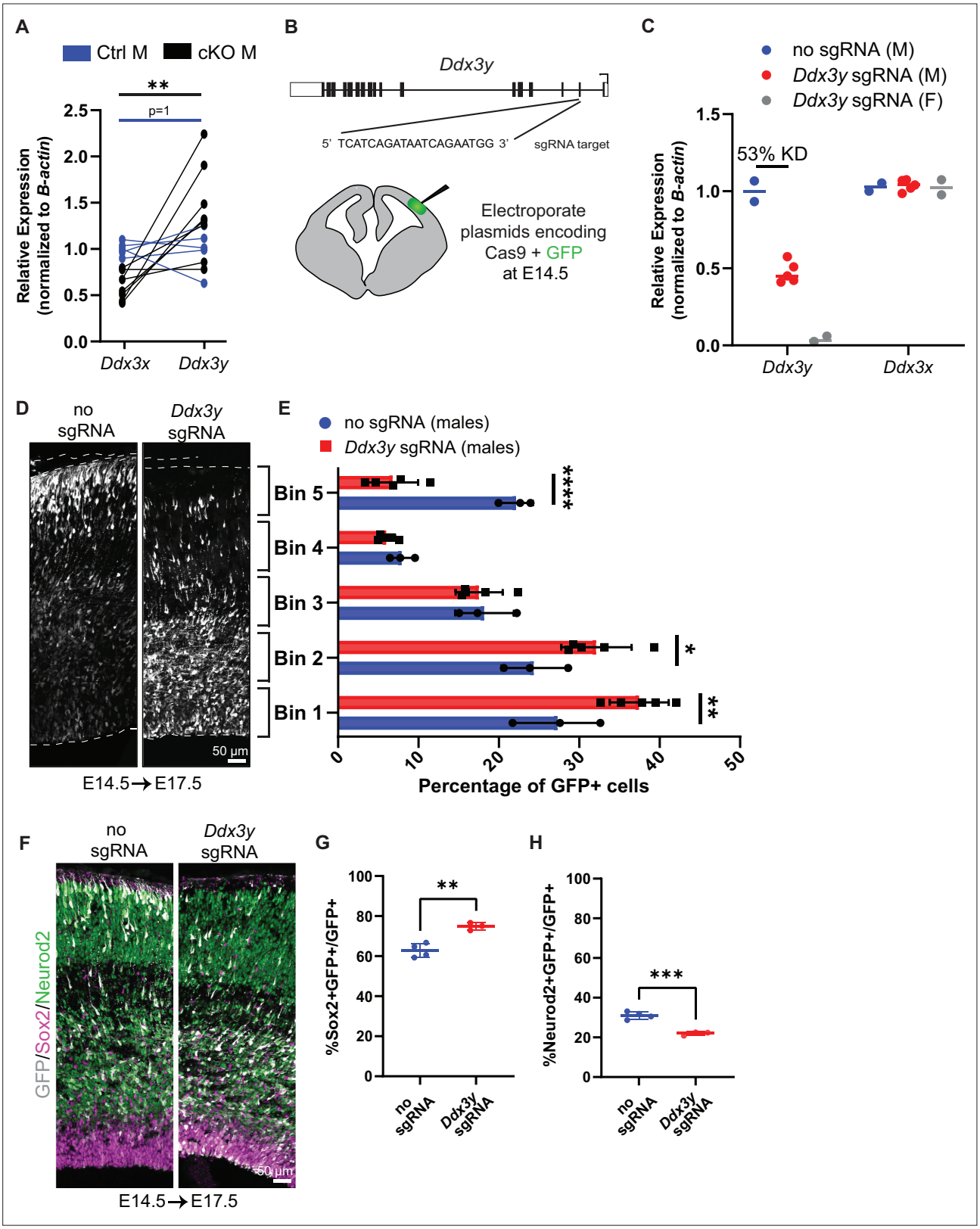

Figure 2. *Ddx3x* knockout is sexually dimorphic and *Ddx3y* phenocopies *Ddx3x* loss. (**A**) RT-qPCR quantification of *Ddx3x* and *Ddx3y* mRNA levels in FACS-isolated Tdtomato + cells from cKO male E11.5 cortices. n=5–8 embryos/condition. (**B**) Schematic of *Ddx3y* CRISPR sgRNA electroporation of E14.5 brain. (**C**) RT-qPCR quantification of *Ddx3y* and *Ddx3x* levels in GFP +FACS-isolated cells from E17.5 male and female mice electroporated with pCAG-GFP and either no sgRNA or *Ddx3y* sgRNA. n=2–5 embryos/condition. (**D**) Representative sections of E17.5 male brains electroporated at E14.5

Figure 2 continued on next page

Figure 2 continued

with pCAG-GFP and either no sgRNA or *Ddx3y* sgRNA and stained with anti-GFP (grey). Dotted lines, ventricular and pial surfaces; brackets delineate equivalently sized bins. (**E**) Quantification of distribution of GFP + cells. n=3–5 embryos/condition. (**F**) Same as (**D**), but sections were stained with anti-GFP (grey), Sox2 (magenta), and Neurod2 (green). (**G, H**) Quantification of GFP co-localization with Sox2 (**G**) or Neurod2 (**H**). n=3–5 embryos/condition. Scale bars, indicated. Error bars, S.D. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Student's paired, two-tailed t-test (**A**), Two-way ANOVA with Sidak's (**E**), Student's unpaired, two-tailed t-test (**G, H**).

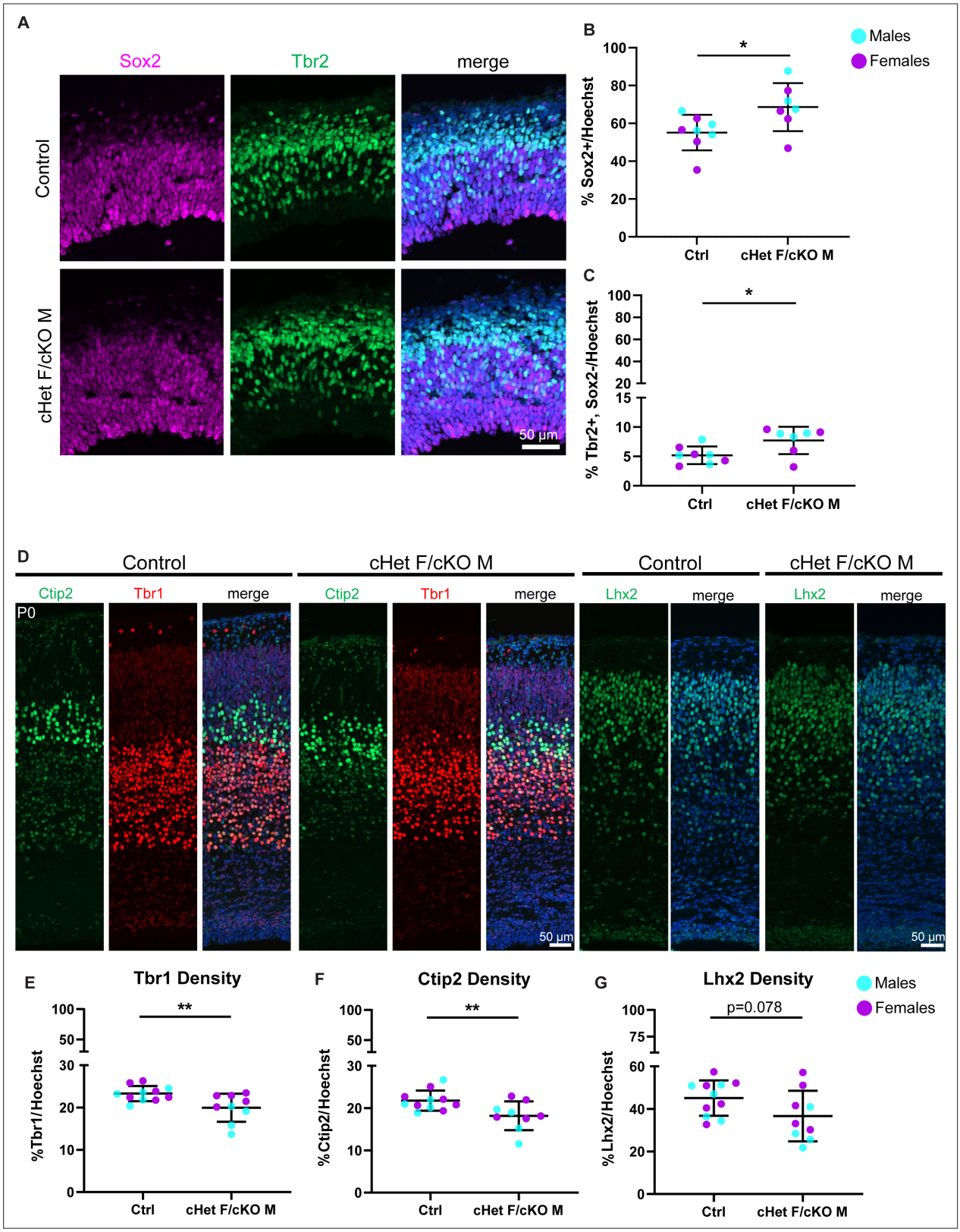

Figure 3. *Ddx3x* depletion leads to more RGCs and mature IPs, and fewer excitatory neurons across laminar layers. (**A**) Representative sections from E14.5 cortices stained with Sox2 (magenta) and Tbr2 (green) (control M and cKO M shown). (**B, C**) Quantification of density of Sox2+ (RGCs) (**B**) and Tbr2 +Sox2- (mature IPs) (**C**) cells relative to all cells (Hoechst) at E14.5. n=7–8 embryos/condition. (**D**) Representative sections stained with Ctip2 (green), Tbr1 (red), and Lhx2 (green) from P0 control and cHet F/cKO M cortices (control M and cKO M shown). (**E–G**) Quantification of laminar marker density

Figure 3 continued on next page

Figure 3 continued

for Tbr1 (**E**), Ctip2 (**F**), and Lhx2 (**G**) relative to all cells (Hoechst). n=8–10 embryos/condition. Scale bars, indicated. Error bars, S.D. *p<0.05, **p<0.01. Student's unpaired, two-tailed t-test (**B, C, E–G**).

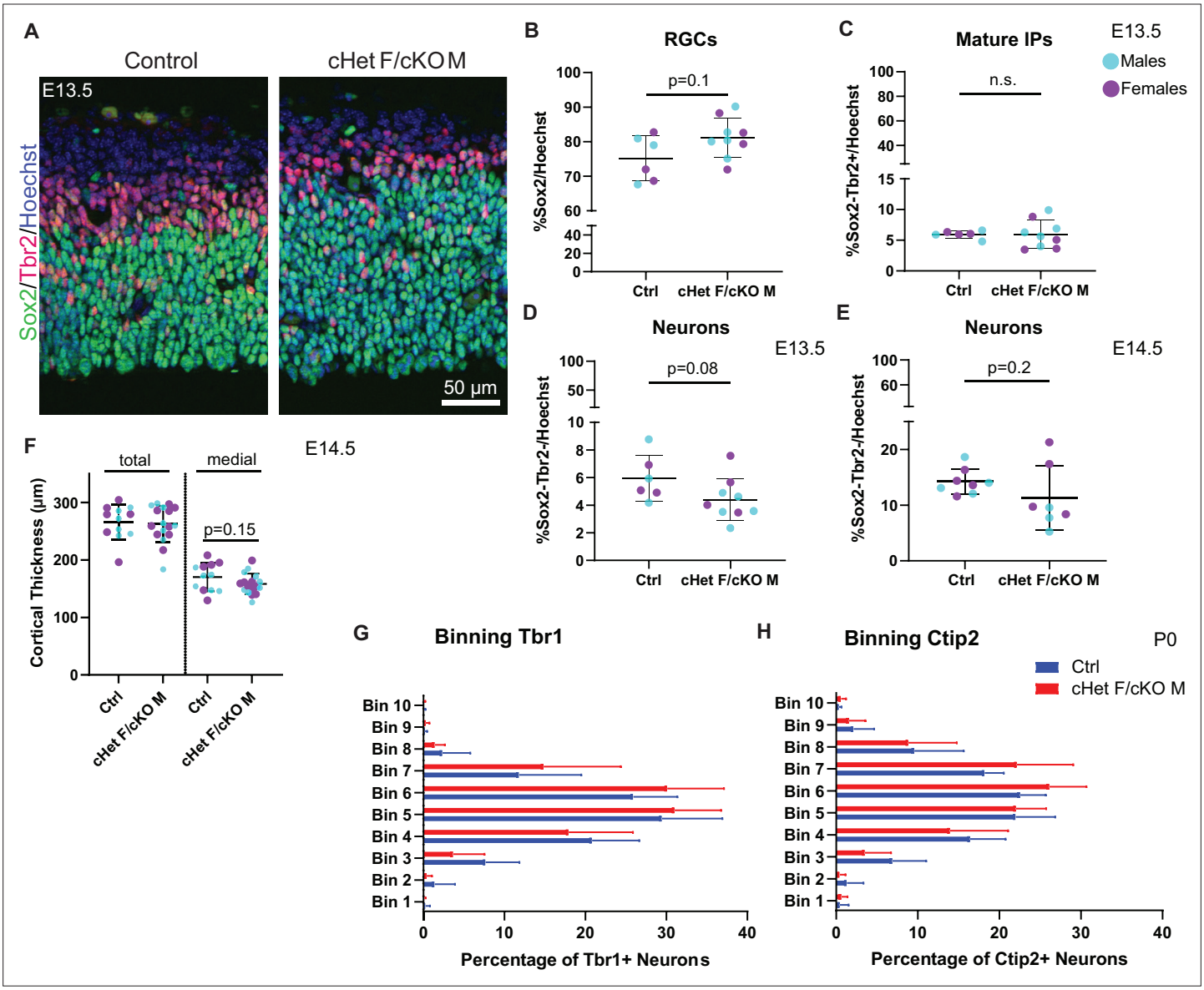

Figure 3—figure supplement 1. *Ddx3x* depletion leads to more progenitors and less neurons at E13.5, but does not affect cortical thickness or laminar position of neurons. (**A**) Representative coronal sections from control and cHet F/cKO M at E13.5 immunostained with Sox2 (green), Tbr2 (magenta) and Hoechst (control M and cKO M shown). (**B–E**) Quantification of RGCs (Sox2+) (**B**), mature IPs (Sox2-,Tbr2+) (**C**), putative neurons (Sox2-Tbr2-), density relative to Hoechst in control and cHet F/cKO M at E13.5 (**D**). n=6–9 embryos/condition. Sox2-Tbr2- density relative to Hoechst in control and cHet F/cKO M at E14.5. n=7–8 embryos/condition (**E**). (**F**) Quantification of cortical thickness at E14.5 in control and cHet F/cKO M measured medially and laterally (total) or just medially. n=12–17 embryos/condition. (**G, H**) Quantification of the distribution of Tbr1+ (**G**) and Ctip2+ (**H**) cells in control and cHet F/cKO M at P0. Student's unpaired, two-tailed t-test (**B–F**), Two-way ANOVA with Sidak's (**G, H**).

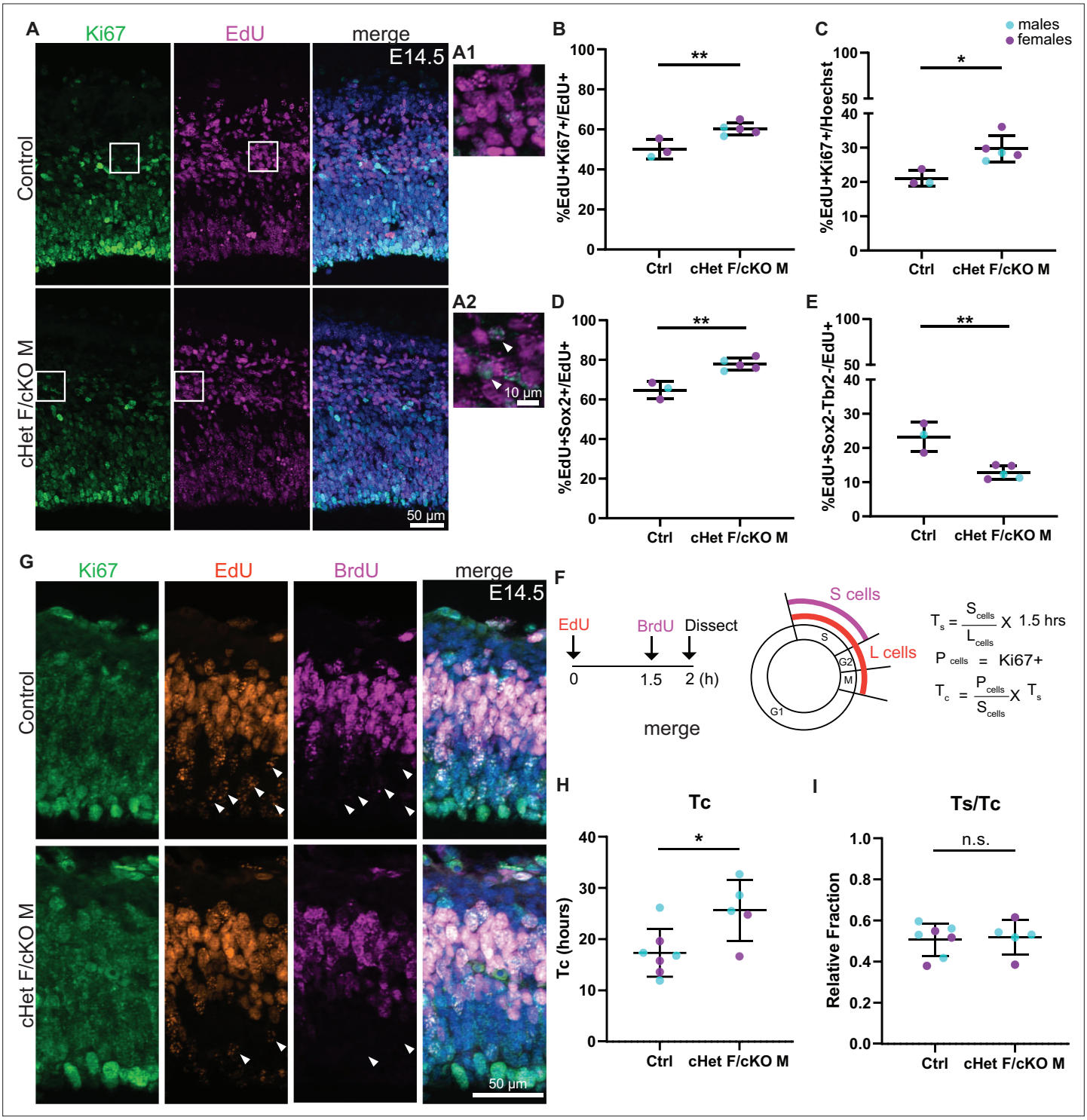

Figure 4. *Ddx3x* depletion impairs progenitor cell cycle exit and prolongs cell cycle duration. (**A**) Representative sections stained with Ki67 (green) and EdU (magenta) from E14.5 control and cHet F/cKO M mice (control F and cHet F shown) pulsed with EdU at E13.5, and higher magnification insets (A1, A2). (**B, C**) Quantification of Ki67 +EdU + relative to EdU + cells (**B**) and all cells (Hoechst, **C**). n=3–5 embryos/condition. (**D, E**) Quantification of EdU +Sox2+ (**D**) and EdU +Sox2-Tbr2- cells (**E**) relative to all EdU + cells. n=3–5 embryos/condition. (**F**) Schematic illustrating the semi-cumulative labeling paradigm and cell cycle formulas. ***Figure 4F*** has been adapted from Figure 4J from ***Boyd et al., 2015***. (**G**) Representative medial sections of E14.5 control and cHet F/cKO M brains stained with Ki67 (green), EdU (red) and BrdU (magenta) and pulsed with EdU and BrdU (control M and cKO M shown). Arrows indicate EdU +BrdU cells (i.e: leaving cells). (**H**) Quantification of cell cycle duration (Tc) in control and cHet F/cKO M. n=5–7 embryos/condition. (**I**) Quantification of Ts/Tc in control and cHet F/cKO M. n=5–7 embryos/condition. Scale bars, indicated. Error bars, S.D. *p<0.05, **p<0.01. Student's unpaired, two-tailed t-test (**B–E, H, I**).

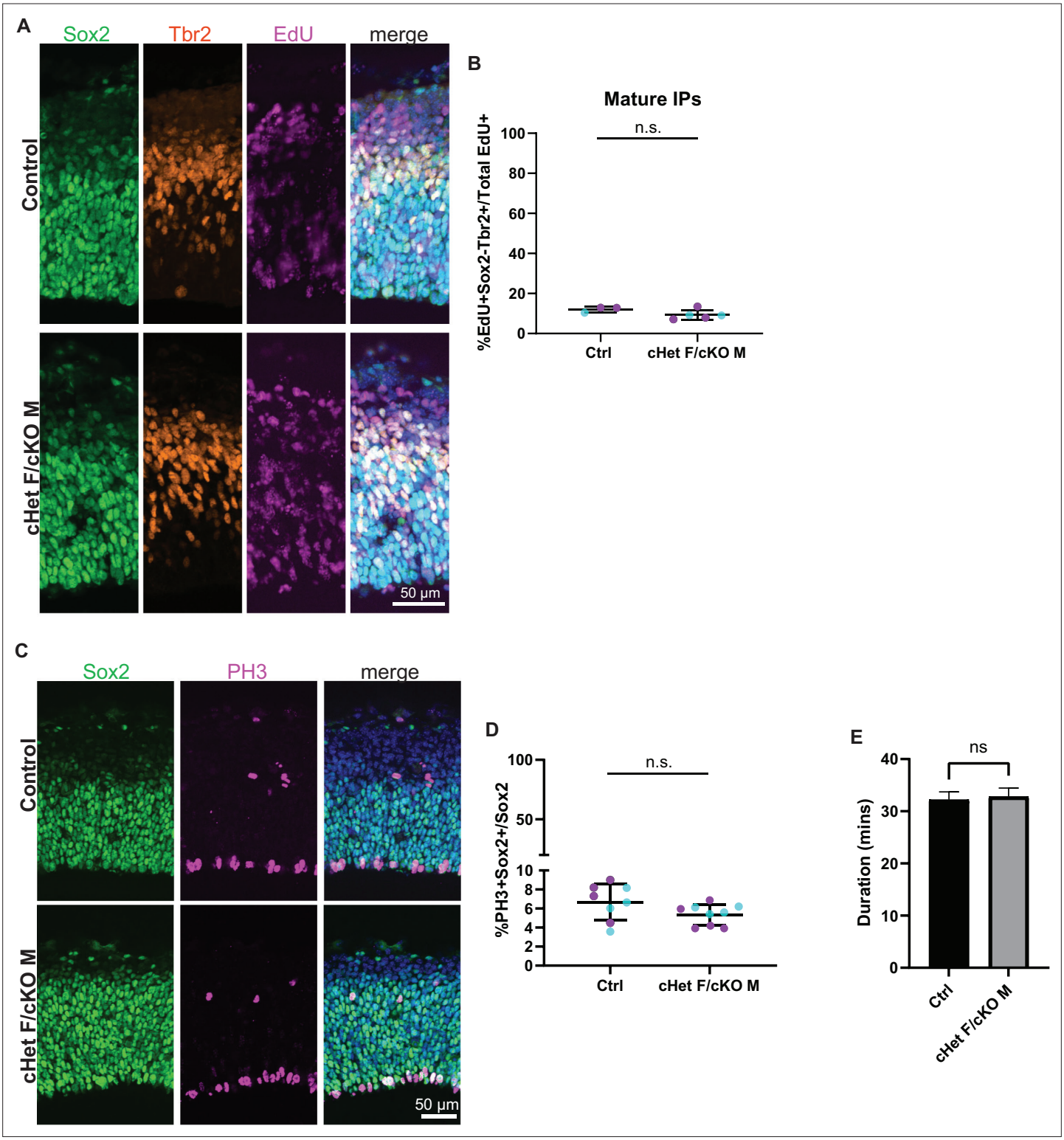

Figure 4—figure supplement 1. *Ddx3x* depletion prolongs cell cycle duration in RGCs and immature IPs but does not affect mitosis duration. (**A**) Representative coronal sections from control and cHet F/cKO M at E14.5 immunostained with Sox2 (green), Tbr2 (red), EdU (magenta) and Hoechst (control F and cHet F shown). (**B**) Quantification of density of mature IPs (EdU+Sox2-Tbr2+) relative to total EdU in control and cHet F/cKO M at E14.5. n=3–5 embryos/condition. (**C**) Representative coronal sections from control and cHet F/cKO M at E14.5 immunostained with Sox2 (green), PH3 (magenta), and Hoechst (control M and cKO M shown). (**D**) Quantification of PH3+Sox2+/Sox2+ in control and cHet F/cKO M. n=8–9 embryos/condition. (**E**) Quantification of mitosis duration in control and cHet F/cKO M at E14.5 from live imaging analysis. n=>100 cells/condition/trial with three trials. Student's unpaired, two-tailed t-test (**B, D, E**).

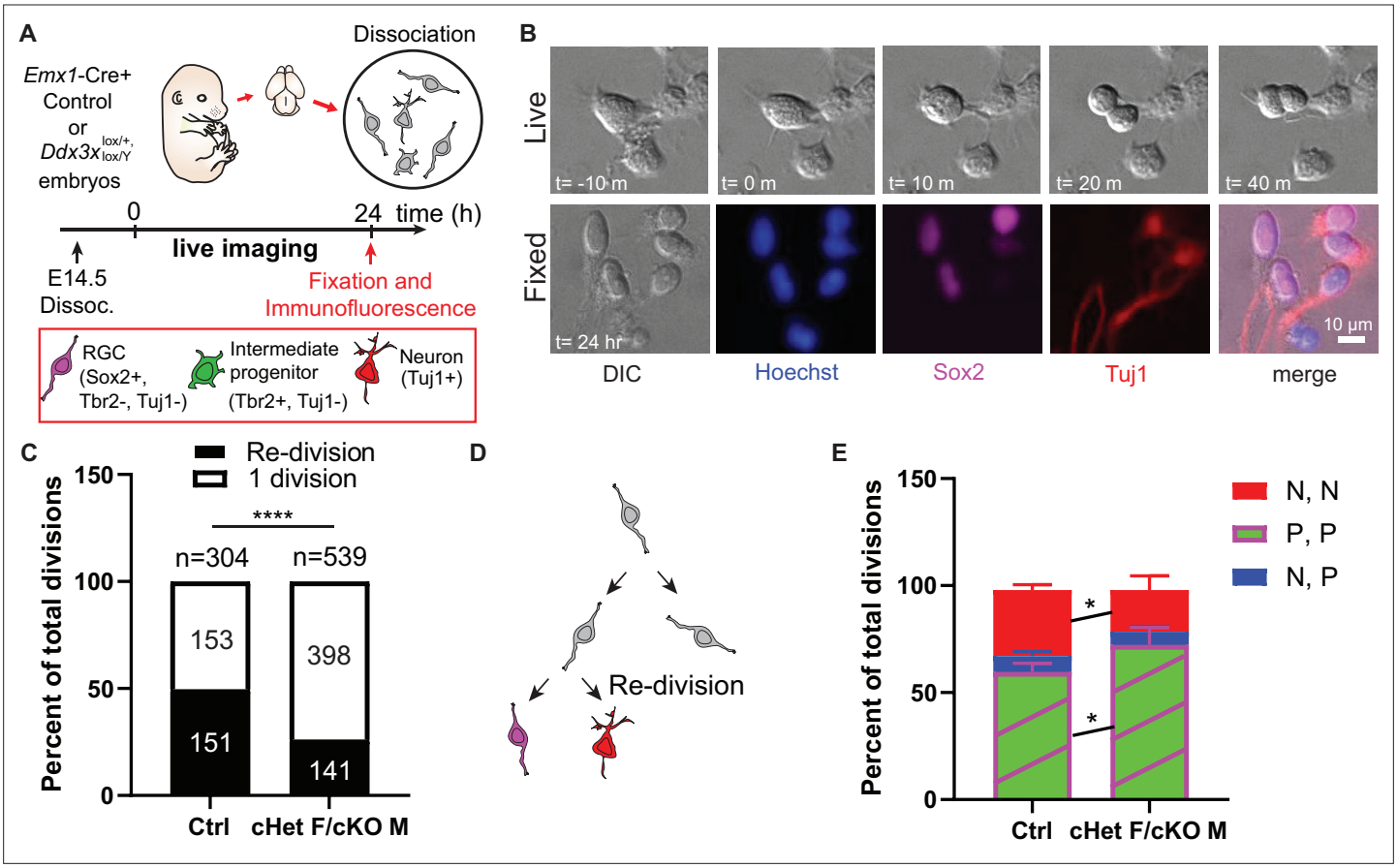

Figure 5. *Ddx3x*-depleted progenitors exhibit prolonged cell division and more proliferative divisions at the expense of neurogenic divisions. (**A**) Live imaging paradigm for monitoring cell fate. ***Figure 5A*** has been adapted from Figure 3A and E from ***Pilaz et al., 2016***. (**B**) Live imaging DIC snapshots at indicated t=minutes or hours, and fixed images stained with indicated markers. (**C**) Quantification of re-divisions (black) and 1 division (white) in control and cHet F/cKO M. n=304 (control) and 539 (cHet F/cKO M) total cells. (**D**) Schematic illustrating an example of a re-division. (**E**) Quantification of cell fate for P,P divisions (2 Sox2+ RGCs, or 2 Tbr2+ IPs, or 1 Sox2+ RGC and 1 Tbr2+Tuj1- IP); P, N divisions (1 Tuj1+ neuron and either 1 Sox2+ RGC or 1 Tbr2+ IP); N, N divisions (2 Tuj1+ neurons). n=>70 cells/condition/trial with three trials. Scale bars, indicated. Error bars, S.D. *p<0.05, ****p<0.0001. Two-tailed Fisher's exact test (**C**), Two-way ANOVA with Sidak's correction (**E**).

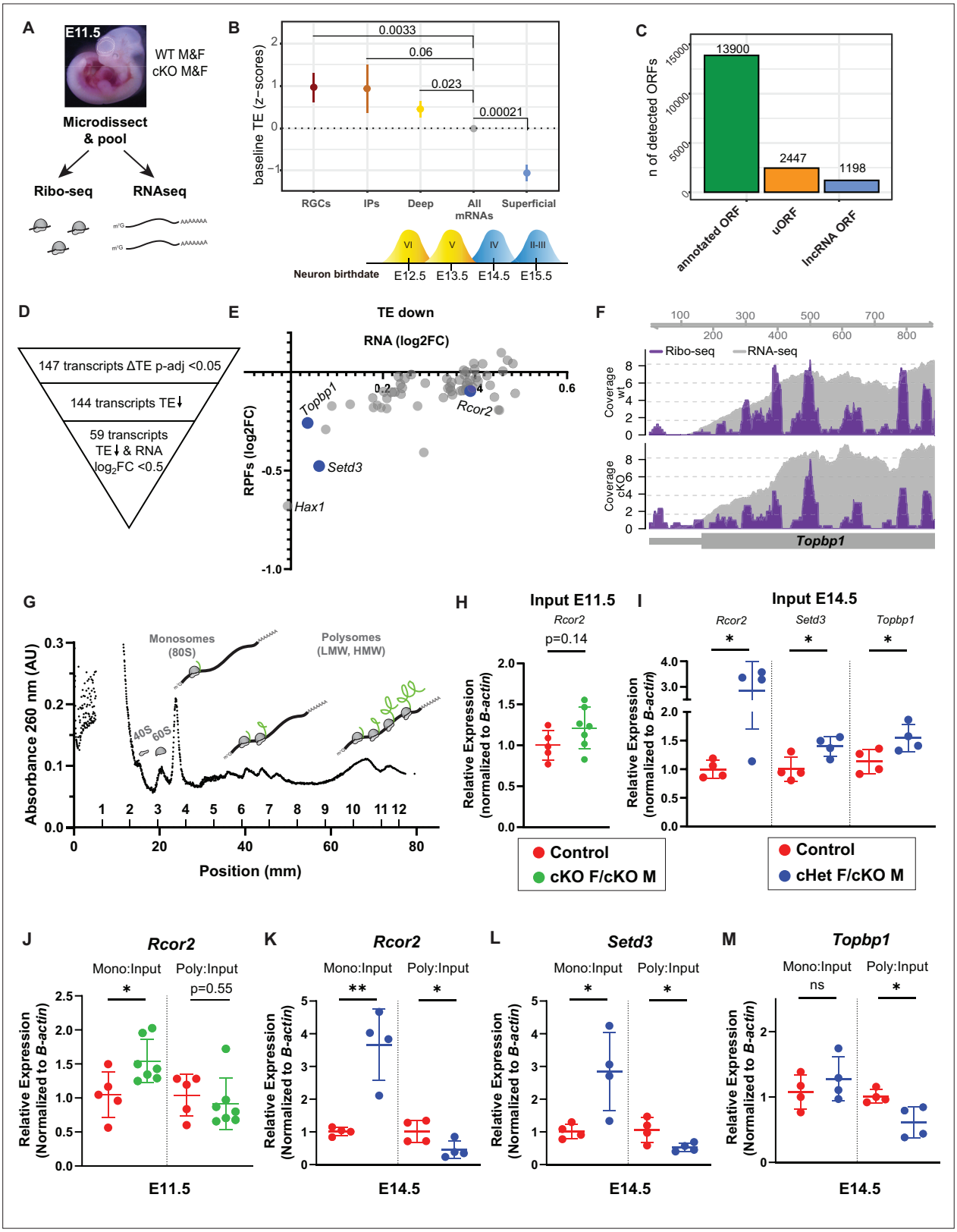

Figure 6. Ribosome Profiling in embryonic brains uncovers the E11.5 translatome including DDX3X-dependent translation targets. (**A**) Experimental paradigm for Ribo-seq and RNAseq of E11.5 cortices from control and cKO mice. n=3/sex/condition with four embryos pooled per n. (**B**) TE of transcripts enriched in RGCs, IPs, deep layer neurons (VI-V) and superficial layer neurons (IV-II) relative to all other mRNAs (TPM >10). Birthdates for laminar layers are indicated below. See ***Supplementary file 3*** for exact transcripts. (**C**) ORFquant analysis of wildtype Ribo-seq data showing

Figure 6 continued on next page

Figure 6 continued

identification of annotated ORFs and uORFs in protein-coding and non-coding isoforms. (**D**) Schematic illustrating how DDX3X-dependent targets were prioritized. (**E**) Scatter plot of RPFs log2FC versus RNA log2FC for 59 DDX3X-dependent targets with significantly lower TE. Putative Ribo-seq targets selected for validation are highlighted in blue. (**F**) IGV screenshots illustrating RNAseq reads (gray) and RPFs (Ribo-seq; purple) for *Topbp1* in cKO mice relative to control. (**G**) Representative trace from polysome fractionation of E14.5 cortical lysate. (**H–M**) RT-qPCR quantification of mRNA levels for Ribo-seq candidates in input samples at E11.5 (**H**) and at E14.5 (**I**), and monosome and polysome fractions at E11.5 (**J**) and E14.5 (**K–M**). n=5–7/condition (**H, J**) and 4/condition (**I, K–M**) with two embryos pooled per n. Error bars, S.D. *p<0.05, **p<0.01. Two-sided Wilcoxon test (**B**), Student's unpaired, two-tailed t-test (**H–M**).

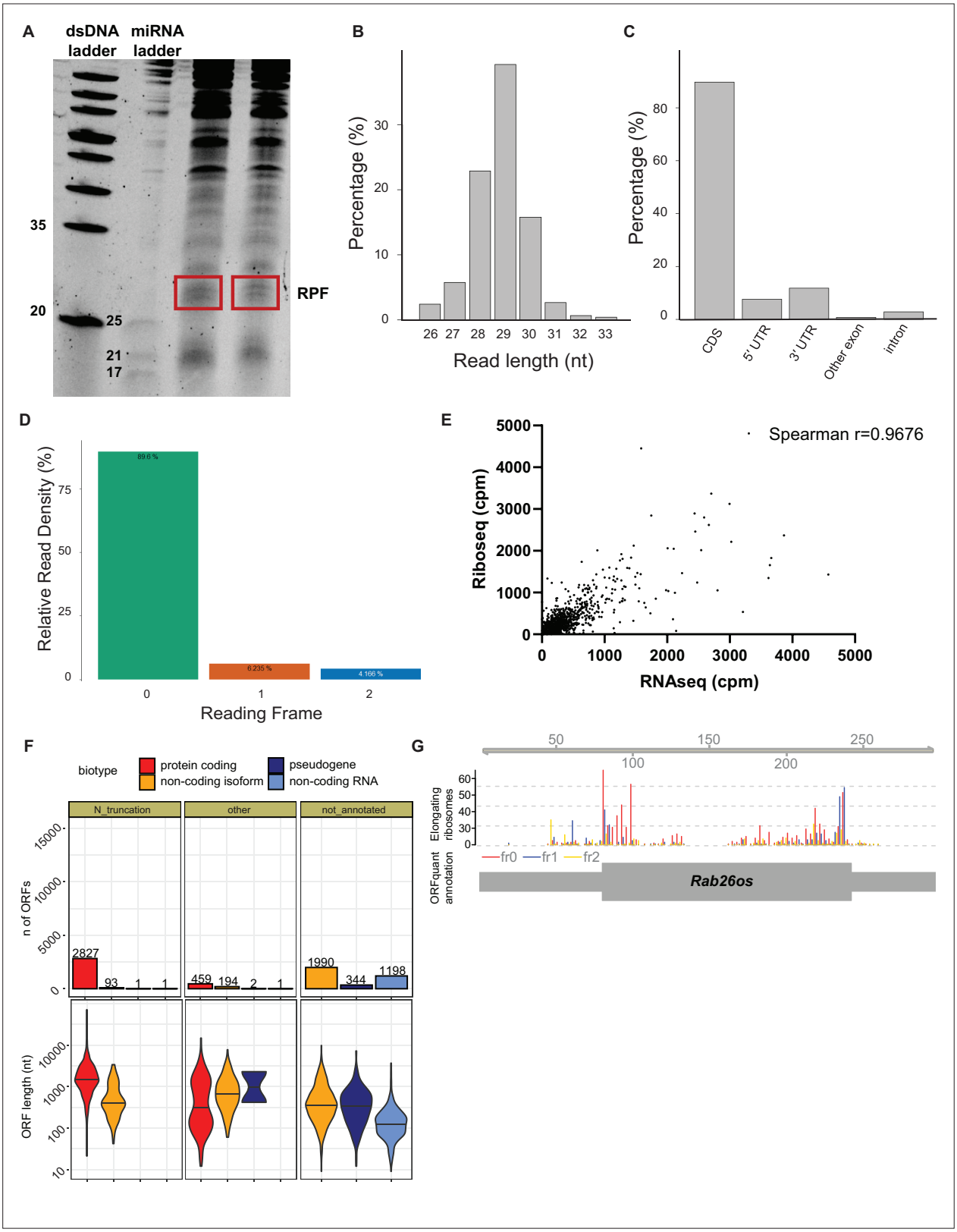

Figure 6—figure supplement 1. Quality Control Assessment of Ribosome Profiling in *Ddx3x* cKO mice. (**A**) Representative denaturing urea gel of embryonic cortices treated with RNase I illustrating RPFs (red box). (**B–D**) RibosomeProfilingQC assessment of deep sequencing of cDNA libraries showing read length distribution (**B**), percent of reads mapping to CDS and UTRs, etc (**C**), and reading frame (**D**). (**E**) Comparison of RNAseq cpm and Riboseq cpm using all reads from all transcripts from WT data (excluding non-polyA histone and multi-mapping ribosomal genes); Spearman *r*=0.9676.

Figure 6—figure supplement 1 continued on next page

Figure 6—figure supplement 1 continued

Five transcripts were omitted when reducing axes for readability. (**F**) *De novo* identification of translated ORFs, including number of detected ORFs with their length (in nucleotides) for different ORF categories and annotated biotypes. (**G**) A novel translated ORF in the lncRNA *Rab26os* showing the P-sites position colored by frame (middle), and ORF quant-derived annotation (bottom).

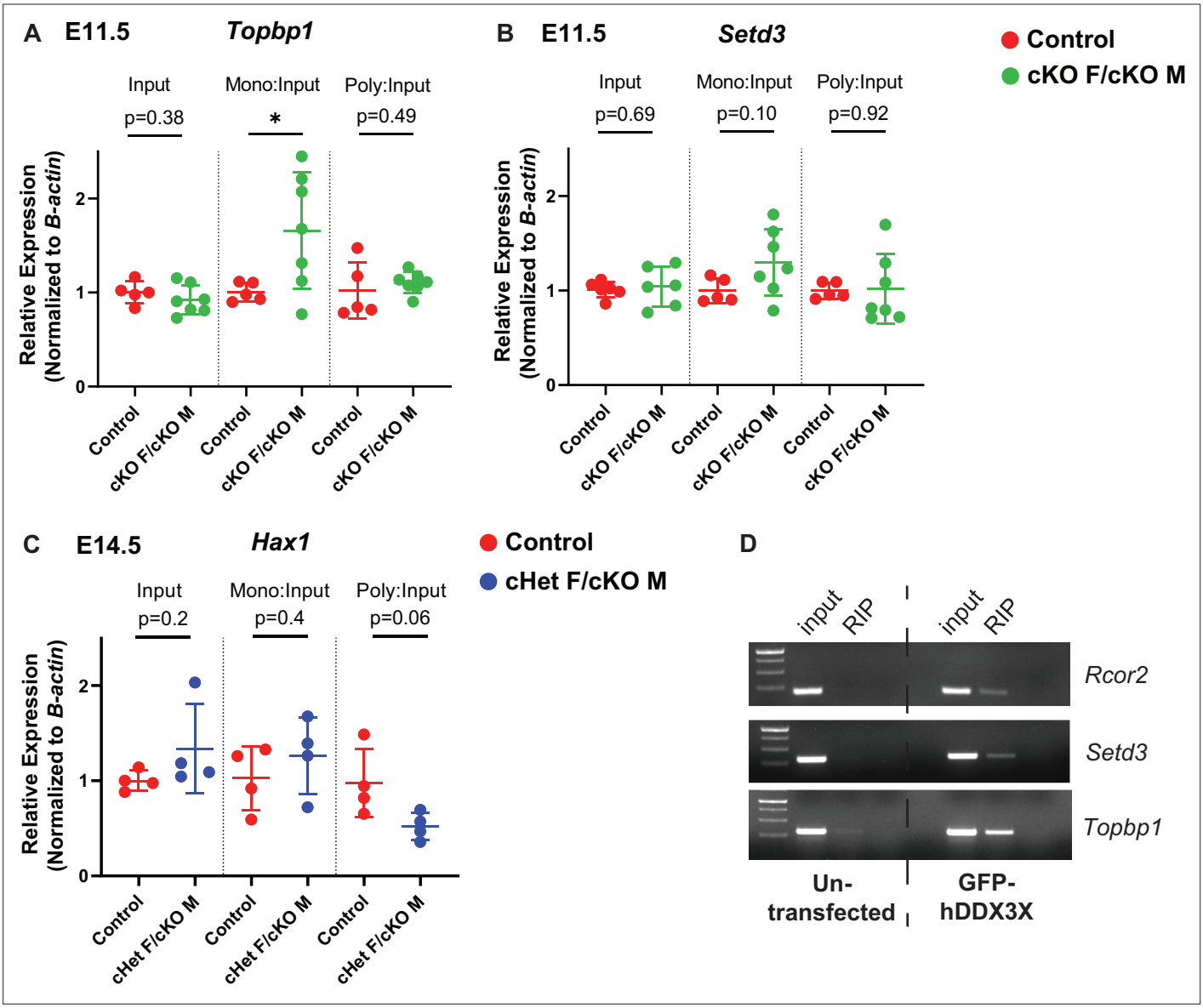

Figure 6—figure supplement 2. Polysome fractionation and RNA immunoprecipitations showing DDX3X targets in the cortex. (**A–C**) RT-qPCR quantification of mRNA levels for Ribo-seq candidates in input samples, monosome and polysome fractions at E11.5 for *Topbp1* (**A**) and *Setd3* (**B**) and at E14.5 for *Hax1* (**C**). (**D**) Representative gels of RNA immunoprecipitation of DDX3X translation targets, *Rcor2*, *Setd3*, and *Topbp1*. n=5–7 embryos/condition (**A, B**), 4 embryos/condition (**C**), 3 biological replicates (**D**). Student's unpaired, two-tailed t-test (**A–C**).

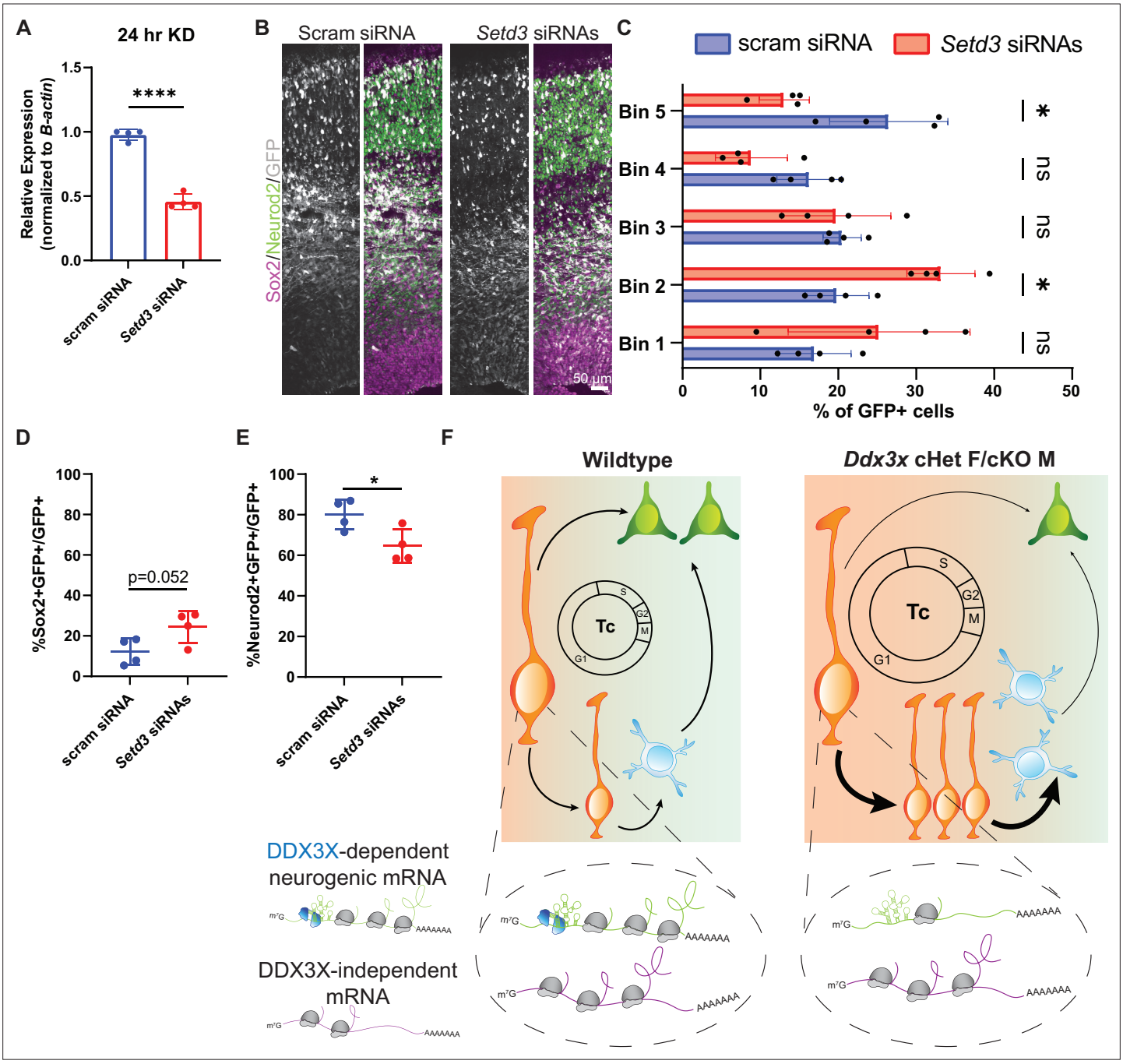

Figure 7. DDX3X-dependent translation target, *Setd3*, is required for neurogenesis. (**A**) RT-qPCR quantification of *Setd3* knockdown in N2A cells. n=4/condition with two independent trials. (**B**) Representative sections of E17.5 brains from mice electroporated at E14.5 with pCAG-GFP and scrambled or *Setd3* siRNAs and immunostained with GFP (grey), Sox2 (magenta), Neurod2 (green). (**C**) Quantification of distribution of GFP-positive cells in 5 even bins of cortex. n=4 embryos/condition. (**D, E**) Quantification of GFP co-localization with Sox2+ (**D**) and Neurod2+ (**E**) cells. n=4 embryos/condition. (**F**) Schematic model summarizing how loss of DDX3X-dependent translation impairs neurogenesis. Scale bars, indicated. Error bars, S.D. *p<0.05, ****p<0.0001. Student's unpaired, two-tailed t-test (**A, D, E**), Two-way ANOVA with Sidak's correction (**C**).